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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US99/15870 <b>(22) International Filing Date:</b> 13 July 1999 (13.07.99)  <b>(30) Priority Data:</b> 60/092,671 13 July 1998 (13.07.98) US  <b>(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application</b> US 60/092,671 (CIP) Filed on 13 July 1998 (13.07.98)  <b>(71) Applicant (for all designated States except US):</b> CELLOMICS, INC. [US/US]; 635 William Pitt Way, Pittsburgh, PA 15238 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> RUBIN, Richard, A. [US/US]; 35 William Pitt Way, Pittsburgh, PA 15238 (US). GIULIANO, Ken, A. [US/US]; 635 William Pitt Way, Pittsburgh, PA 15238 (US). GOUGH, Albert [US/US]; 635 William Pitt Way, Pittsburgh, PA 15238 (US). DUNLAY, Terry [US/US]; 635 William Pitt Way, Pittsburgh, PA 15238 (US).	<b>(74) Agent:</b> HARPER, David, S.; McDonnell Boehnen Hulbert & Berghoff, 300 South Wacker Drive, Chicago, IL 60606 (US).  <b>(81) Designated States:</b> AU, CA, JP, MX, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>	
<b>(54) Title:</b> A SYSTEM FOR CELL-BASED SCREENING  <b>(57) Abstract</b>  The present invention provides systems, methods, and screens to measure receptor internalization in a single step with appropriate automation and throughput. This approach involves luminescent labeling of the receptor of interest and the automated measurement of receptor internalization to a perinuclear location.		

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**A SYSTEM FOR CELL-BASED SCREENING**  
**(Case No. 97,022-D2)**

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**Cross Reference**

This application is a continuation-in-part of U.S. Applications for Patent S/N 60/092,671, filed on July 13, 1998.

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**Field of The Invention**

This invention is in the field of fluorescence-based cell and molecular biochemical assays for drug discovery.

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**Background of the Invention**

Drug discovery, as currently practiced in the art, is a long, multiple step process involving identification of specific disease targets, development of an assay based on a specific target, validation of the assay, optimization and automation of the assay to produce a screen, high throughput screening of compound libraries using the assay to identify "hits", hit validation and hit compound optimization. The output of this process is a lead compound that goes into pre-clinical and, if validated, eventually into clinical trials. In this process, the screening phase is distinct from the assay development phases, and involves testing compound efficacy in living biological systems.

25

Historically, drug discovery is a slow and costly process, spanning numerous years and consuming hundreds of millions of dollars per drug created. Developments in the areas of genomics and high throughput screening have resulted in increased capacity and efficiency in the areas of target identification and volume of compounds

screened. Significant advances in automated DNA sequencing, PCR application, positional cloning, hybridization arrays, and bioinformatics have greatly increased the number of genes (and gene fragments) encoding potential drug screening targets. However, the basic scheme for drug screening remains the same.

5        Validation of genomic targets as points for therapeutic intervention using the existing methods and protocols has become a bottleneck in the drug discovery process due to the slow, manual methods employed, such as *in vivo* functional models, functional analysis of recombinant proteins, and stable cell line expression of candidate genes. Primary DNA sequence data acquired through automated sequencing does not  
10   permit identification of gene function, but can provide information about common "motifs" and specific gene homology when compared to known sequence databases. Genomic methods such as subtraction hybridization and RADE (rapid amplification of differential expression) can be used to identify genes that are up or down regulated in a disease state model. However, identification and validation still proceed down the same  
15   pathway. Some proteomic methods use protein identification (global expression arrays, 2D electrophoresis, combinatorial libraries) in combination with reverse genetics to identify candidate genes of interest. Such putative "disease associated sequences" or DAS isolated as intact cDNA are a great advantage to these methods, but they are identified by the hundreds without providing any information regarding type, activity,  
20   and distribution of the encoded protein. Choosing a subset of DAS as drug screening targets is "random", and thus extremely inefficient, without functional data to provide a mechanistic link with disease. It is necessary, therefore, to provide new technologies to rapidly screen DAS to establish biological function, thereby improving target validation and candidate optimization in drug discovery.

There are three major avenues for improving early drug discovery productivity. First, there is a need for tools that provide increased information handling capability. Bioinformatics has blossomed with the rapid development of DNA sequencing systems and the evolution of the genomics database. Genomics is beginning to play a critical  
5 role in the identification of potential new targets. Proteomics has become indispensable in relating structure and function of protein targets in order to predict drug interactions. However, the next level of biological complexity is the cell. Therefore, there is a need to acquire, manage and search multi-dimensional information from cells. Secondly, there is a need for higher throughput tools. Automation is a key to improving  
10 productivity as has already been demonstrated in DNA sequencing and high throughput primary screening. The instant invention provides for automated systems that extract multiple parameter information from cells that meet the need for higher throughput tools. The instant invention also provides for miniaturizing the methods, thereby  
— allowing increased throughput, while decreasing the volumes of reagents and test  
15 compounds required in each assay.

Radioactivity has been the dominant read-out in early drug discovery assays. However, the need for more information, higher throughput and miniaturization has caused a shift towards using fluorescence detection. Fluorescence-based reagents can yield more powerful, multiple parameter assays that are higher in throughput and  
20 information content and require lower volumes of reagents and test compounds. Fluorescence is also safer and less expensive than radioactivity-based methods.

Screening of cells treated with dyes and fluorescent reagents is well known in the art. There is a considerable body of literature related to genetic engineering of cells to produce fluorescent proteins, such as modified green fluorescent protein (GFP), as a

reporter molecule. Some properties of wild-type GFP are disclosed by Morise et al. (*Biochemistry* 13 (1974), p. 2656-2662), and Ward et al. (*Photochem. Photobiol.* 31 (1980), p. 611-615). The GFP of the jellyfish *Aequorea victoria* has an excitation maximum at 395 nm and an emission maximum at 510 nm, and does not require an exogenous factor for fluorescence activity. Uses for GFP disclosed in the literature are widespread and include the study of gene expression and protein localization (Chalfie et al., *Science* 263 (1994), p. 12501-12504)), as a tool for visualizing subcellular organelles (Rizzuto et al., *Curr. Biology* 5 (1995), p. 635-642)), visualization of protein transport along the secretory pathway (Kaether and Gerdes, *FEBS Letters* 369 (1995), p. 267-271)), expression in plant cells (Hu and Cheng, *FEBS Letters* 369 (1995), p. 331-334)) and *Drosophila* embryos (Davis et al., *Dev. Biology* 170 (1995), p. 726-729)), and as a reporter molecule fused to another protein of interest (U. S. Patent 5,491,084). Similarly, WO96/23898 relates to methods of detecting biologically active substances affecting intracellular processes by utilizing a GFP construct having a protein kinase activation site. This patent, and all other patents referenced in this application are incorporated by reference in their entirety

Numerous references are related to GFP proteins in biological systems. For example, WO 96/09598 describes a system for isolating cells of interest utilizing the expression of a GFP like protein. WO 96/27675 describes the expression of GFP in plants. WO 95/21191 describes modified GFP protein expressed in transformed organisms to detect mutagenesis. U. S. Patents 5,401,629 and 5,436,128 describe assays and compositions for detecting and evaluating the intracellular transduction of an extracellular signal using recombinant cells that express cell surface receptors and

contain reporter gene constructs that include transcriptional regulatory elements that are responsive to the activity of cell surface receptors.

Performing a screen on many thousands of compounds requires parallel handling and processing of many compounds and assay component reagents. Standard high throughput screens ("HTS") use mixtures of compounds and biological reagents along with some indicator compound loaded into arrays of wells in standard microtiter plates with 96 or 384 wells. The signal measured from each well, either fluorescence emission, optical density, or radioactivity, integrates the signal from all the material in the well giving an overall population average of all the molecules in the well.

10 Science Applications International Corporation (SAIC) 130 Fifth Avenue, Seattle, WA. 98109) describes an imaging plate reader. This system uses a CCD camera to image the whole area of a 96 well plate. The image is analyzed to calculate the total fluorescence per well for all the material in the well.

Molecular Devices, Inc. (Sunnyvale, CA) describes a system (FLIPR) which uses low angle laser scanning illumination and a mask to selectively excite fluorescence within approximately 200 microns of the bottoms of the wells in standard 96 well plates in order to reduce background when imaging cell monolayers. This system uses a CCD camera to image the whole area of the plate bottom. Although this system measures signals originating from a cell monolayer at the bottom of the well, the signal measured is averaged over the area of the well and is therefore still considered a measurement of the average response of a population of cells. The image is analyzed to calculate the total fluorescence per well for cell-based assays. Fluid delivery devices have also been incorporated into cell based screening systems, such as the FLIPR



system, in order to initiate a response, which is then observed as a whole well population average response using a macro-imaging system.

In contrast to high throughput screens, various high-content screens ("HCS") have been developed to address the need for more detailed information about the temporal-spatial dynamics of cell constituents and processes. High-content screens automate the extraction of multicolor fluorescence information derived from specific fluorescence-based reagents incorporated into cells (Giuliano and Taylor (1995), *Curr. Op. Cell Biol.* 7:4; Giuliano et al. (1995) *Ann. Rev. Biophys. Biomol. Struct.* 24:405). Cells are analyzed using an optical system that can measure spatial, as well as temporal dynamics. (Farkas et al. (1993) *Ann. Rev. Physiol.* 55:785; Giuliano et al. (1990) In *Optical Microscopy for Biology*. B. Herman and K. Jacobson (eds.), pp. 543-557. Wiley-Liss, New York; Hahn et al (1992) *Nature* 359:736; Waggoner et al. (1996) *Hum. Pathol.* 27:494). The concept is to treat each cell as a "well" that has spatial and temporal information on the activities of the labeled constituents.

The types of biochemical and molecular information now accessible through fluorescence-based reagents applied to cells include ion concentrations, membrane potential, specific translocations, enzyme activities, gene expression, as well as the presence, amounts and patterns of metabolites, proteins, lipids, carbohydrates, and nucleic acid sequences (DeBiasio et al., (1996) *Mol. Biol. Cell.* 7:1259; Giuliano et al., (1995) *Ann. Rev. Biophys. Biomol. Struct.* 24:405; Heim and Tsien, (1996) *Curr. Biol.* 6:178).

High-content screens can be performed on either fixed cells, using fluorescently labeled antibodies, biological ligands, and/or nucleic acid hybridization probes, or live

cells using multicolor fluorescent indicators and "biosensors." The choice of fixed or live cell screens depends on the specific cell-based assay required.

Fixed cell assays are the simplest, since an array of initially living cells in a microtiter plate format can be treated with various compounds and doses being tested, then the cells can be fixed, labeled with specific reagents, and measured. No environmental control of the cells is required after fixation. Spatial information is acquired, but only at one time point. The availability of thousands of antibodies, ligands and nucleic acid hybridization probes that can be applied to cells makes this an attractive approach for many types of cell-based screens. The fixation and labeling steps can be automated, allowing efficient processing of assays.

Live cell assays are more sophisticated and powerful, since an array of living cells containing the desired reagents can be screened over time, as well as space. Environmental control of the cells (temperature, humidity, and carbon dioxide) is required during measurement, since the physiological health of the cells must be maintained for multiple fluorescence measurements over time. There is a growing list of fluorescent physiological indicators and "biosensors" that can report changes in biochemical and molecular activities within cells (Giuliano et al., (1995) *Ann. Rev. Biophys. Biomol. Struct.* 24:405; Hahn et al., (1993) In *Fluorescent and Luminescent Probes for Biological Activity*. W.T. Mason, (ed.), pp. 349-359, Academic Press, San Diego).

The availability and use of fluorescence-based reagents has helped to advance the development of both fixed and live cell high-content screens. Advances in instrumentation to automatically extract multicolor, high-content information has recently made it possible to develop HCS into an automated tool. An article by Taylor,

et al. (*American Scientist* 80 (1992), p. 322-335) describes many of these methods and their applications. For example, Proffitt et. al. (*Cytometry* 24: 204-213 (1996)) describe a semi-automated fluorescence digital imaging system for quantifying relative cell numbers in situ in a variety of tissue culture plate formats, especially 96-well microtiter  
5 plates. The system consists of an epifluorescence inverted microscope with a motorized stage, video camera, image intensifier, and a microcomputer with a PC-Vision digitizer. Turbo Pascal software controls the stage and scans the plate taking multiple images per well. The software calculates total fluorescence per well, provides for daily calibration, and configures easily for a variety of tissue culture plate formats.  
10 Thresholding of digital images and reagents which fluoresce only when taken up by living cells are used to reduce background fluorescence without removing excess fluorescent reagent.

Scanning confocal microscope imaging (Go et al., (1997) *Analytical Biochemistry* 247:210-215; Goldman et al., (1995) *Experimental Cell Research*  
15 221:311-319) and multiphoton microscope imaging (Denk et al., (1990) *Science* 248:73; Gratton et al., (1994) *Proc. of the Microscopical Society of America*, pp. 154-155) are also well established methods for acquiring high resolution images of microscopic samples. The principle advantage of these optical systems is the very shallow depth of focus, which allows features of limited axial extent to be resolved  
20 against the background. For example, it is possible to resolve internal cytoplasmic features of adherent cells from the features on the cell surface. Because scanning multiphoton imaging requires very short duration pulsed laser systems to achieve the high photon flux required, fluorescence lifetimes can also be measured in these systems (Lakowicz et al., (1992) *Anal. Biochem.* 202:316-330; Gerritsen et al. (1997), *J. of*

*Fluorescence* 7:11-15)), providing additional capability for different detection modes. Small, reliable and relatively inexpensive laser systems, such as laser diode pumped lasers, are now available to allow multiphoton confocal microscopy to be applied in a fairly routine fashion.

5       A combination of the biological heterogeneity of cells in populations (Bright, et al., (1989). *J. Cell. Physiol.* 141:410; Giuliano, (1996) *Cell Motil. Cytoskel.* 35:237)) as well as the high spatial and temporal frequency of chemical and molecular information present within cells, makes it impossible to extract high-content information from populations of cells using existing whole microtiter plate readers. No existing high-  
10   content screening platform has been designed for multicolor, fluorescence-based screens using cells that are analyzed individually. Similarly, no method is currently available that combines automated fluid delivery to arrays of cells for the purpose of systematically screening compounds for the ability to induce a cellular response that is identified by HCS analysis, especially from cells grown in microtiter plates.  
15   Furthermore, no method exists in the art combining high throughput well-by-well measurements to identify "hits" in one assay followed by a second high content cell-by-cell measurement on the same plate of only those wells identified as hits.

      The instant invention provides systems, methods, and screens that combine high throughput screening (HTS) and high content screening (HCS) that significantly  
20   improve target validation and candidate optimization by combining many cell screening formats with fluorescence-based molecular reagents and computer-based feature extraction, data analysis, and automation, resulting in increased quantity and speed of data collection, shortened cycle times, and, ultimately, faster evaluation of promising

drug candidates. The instant invention also provides for miniaturizing the methods, thereby allowing increased throughput, while decreasing the volumes of reagents and test compounds required in each assay.

5 **SUMMARY OF THE INVENTION**

The present invention provides fully automated methods for measuring and analyzing cell surface receptor protein internalization during image acquisition. This approach involves fluorescent labeling of the receptor of interest and the automated  
10 measurement of receptor internalization in stimulated cells.

In one aspect of the present invention, methods, computer readable storage medium, and kits are provided for identifying compounds that induce or inhibit internalization of cell surface receptor proteins, comprising treating cells that possess a luminescently-tagged cell surface receptor protein with a test compound, obtaining  
15 luminescent signals from the cells, converting the luminescent signals into digital data, and utilizing the digital data to determine whether the test compound has induced internalization of the luminescently labeled cell surface receptor protein into the cell.

Various preferred embodiments are provided, that allow for improved spatial resolution and quantitation of the stimulatory or inhibitory effect of the test compound  
20 on receptor internalization. In one such embodiment, the extracellular and intracellular domains of a membrane bound receptor protein are each labeled with a distinct luminescent marker, to permit measuring the relative extracellular availability of external and internal domains of membrane receptors.

In another aspect of the invention, combined high throughput and high content  
25 methods and associated computer readable storage medium are provided for identifying

compounds that induce or inhibit internalization of cell surface receptor proteins. In this aspect, cells are treated with a ligand for the receptor protein of interest, which produces a detectable signal upon stimulation of the receptor protein. The cells are then treated with the test compound, and then scanned in a high throughput mode to  
5 identify those cells that exhibit the ligand-induced detectable signal. Subsequently, only those cells that exhibited the detectable signal are scanned in a high content mode, to determine whether the test compound has induced internalization of the luminescently labeled cell surface receptor protein into the cell.

#### 10 **BRIEF DESCRIPTION OF THE DRAWINGS**

**Figure 1** shows a diagram of the components of the cell-based scanning system.

**Figure 2** shows a schematic of the microscope subassembly.

**Figure 3** shows the camera subassembly.

15 **Figure 4** illustrates cell scanning system process.

**Figure 5** illustrates a user interface showing major functions to guide the user.

**Figure 6** is a block diagram of the two platform architecture of the Dual Mode System for Cell Based Screening in which one platform uses a telescope lens to read all wells of a microtiter plate and a second platform that uses a higher magnification lens to read  
20 individual cells in a well.

**Figure 7** is a detail of an optical system for a single platform architecture of the Dual Mode System for Cell Based Screening that uses a moveable 'telescope' lens to read all wells of a microtiter plate and a moveable higher magnification lens to read individual cells in a well.

**Figure 8** is an illustration of the fluid delivery system for acquiring kinetic data on the Cell Based Screening System.

**Figure 9** is a flow chart of processing step for the cell-based scanning system.

**Figure 10 A-J** illustrates the strategy of the Nuclear Translocation Assay.

- 5 **Figure 11** is a flow chart defining the processing steps in the Dual Mode System for Cell Based Screening combining high throughput and high content screening of microtiter plates.

**Figure 12** is a flow chart defining the processing steps in the High Throughput mode of the System for Cell Based Screening.

- 10 **Figure 13** is a flow chart defining the processing steps in the High Content mode of the System for Cell Based Screening.

**Figure 14** is a flow chart defining the processing steps required for acquiring kinetic data in the High Content mode of the System for Cell Based Screening.

- 15 **Figure 15** is a flow chart defining the processing steps performed within a well during the acquisition of kinetic data.

**Figure 16** is an example of data from a known inhibitor of translocation.

**Figure 17** is an example of data from a known stimulator of translocation.

**Figure 18** illustrates data presentation on a graphical display.

- 20 **Figure 19** is an illustration of the data from the High Throughput mode of the System for Cell Based Screening, an example of the data passed to the High Content mode, the data acquired in the high content mode, and the results of the analysis of that data.

**Figure 20** shows the measurement of a drug-induced cytoplasm to nuclear translocation.

**Figure 21** illustrates a graphical user interface of the measurement shown in Figure 20.

Figure 22 illustrates a graphical user interface of the measurement shown in Fig. 20.

Figure 23 is a graph representing the kinetic data obtained from the measurements depicted in Fig. 20.

Figure 24 details a high-content screen of drug-induced apoptosis.

5 Figure 25 is a graphical representation of data from validation runs of the PTHR internalization screen.

Figure 26 is a flow chart for signal processing.

Figure 27 is a flow chart for an autofocusing procedure to be used in signal processing.

Figure 28 is a flow chart for object processing procedure to be used in signal  
10 processing.

Figure 29 shows a representative display of a PC screen showing receptor internalization data displaying the spot count of individual wells.

Figure 30 shows a representative display of a PC screen showing receptor internalization data displayed on a field by field basis.

15 **DETAILED DESCRIPTION OF THE INVENTION**

All cited patents, patent applications and other references are hereby incorporated by reference in their entirety. As used herein, the following terms have the specified meaning:

20 *Markers of cellular domains.* Luminescent probes that have high affinity for specific cellular constituents including specific organelles or molecules. These probes can either be small luminescent molecules or fluorescently tagged macromolecules used as "labeling reagents", "environmental indicators", or "biosensors."

*Labeling reagents.* Labeling reagents include, but are not limited to,  
25 luminescently labeled macromolecules including fluorescent protein analogs and



biosensors, luminescent macromolecular chimeras including those formed with the green fluorescent protein and mutants thereof, luminescently labeled primary or secondary antibodies that react with cellular antigens involved in a physiological response, luminescent stains, dyes, and other small molecules.

5       *Markers of cellular translocations.* Luminescently tagged macromolecules or organelles that move from one cell domain to another during some cellular process or physiological response. Translocation markers can either simply report location relative to the markers of cellular domains or they can also be "biosensors" that report some biochemical or molecular activity as well.

10       *Biosensors.* Macromolecules consisting of a biological functional domain and a luminescent probe or probes that report the environmental changes that occur either internally or on their surface. A class of luminescently labeled macromolecules designed to sense and report these changes have been termed "fluorescent-protein biosensors". The protein component of the biosensor provides a highly evolved  
15       molecular recognition moiety. A fluorescent molecule attached to the protein component in the proximity of an active site transduces environmental changes into fluorescence signals that are detected using a system with an appropriate temporal and spatial resolution such as the cell scanning system of the present invention. Because the modulation of native protein activity within the living cell is reversible, and because  
20       fluorescent-protein biosensors can be designed to sense reversible changes in protein activity, these biosensors are essentially reusable.

*Disease associated sequences ("DAS").* This term refers to nucleic acid sequences identified by standard techniques, such as primary DNA sequence data, genomic methods such as subtraction hybridization and RADE, and proteomic methods in

combination with reverse genetics, as being of drug candidate compounds. The term does not mean that the sequence is only associated with a disease state.

High content screening (HCS) can be used to measure the effects of drugs on complex molecular events such as signal transduction pathways, as well as cell functions including, but not limited to, apoptosis, cell division, cell adhesion, locomotion, exocytosis, and cell-cell communication. Multicolor fluorescence permits multiple targets and cell processes to be assayed in a single screen. Cross-correlation of cellular responses will yield a wealth of information required for target validation and lead optimization.

10 In one aspect of the present invention, a cell screening system is provided comprising a high magnification fluorescence optical system having a microscope objective, an XY stage adapted for holding a plate with an array of locations for holding cells and having a means for moving the plate to align the locations with the microscope objective and a means for moving the plate in the direction to effect  
15 focusing; a digital camera; a light source having optical means for directing excitation light to cells in the array of locations and a means for directing fluorescent light emitted from the cells to the digital camera; and a computer means for receiving and processing digital data from the digital camera wherein the computer means includes: a digital frame grabber for receiving the images from the camera, a display for user interaction  
20 and display of assay results, digital storage media for data storage and archiving, and means for control, acquisition, processing and display of results.

Figure 1 is a schematic diagram of a preferred embodiment of the cell scanning system. An inverted fluorescence microscope is used 1, such as a Zeiss Axiovert inverted fluorescence microscope which uses standard objectives with magnification of

1-100x to the camera, and a white light source (e.g. 100W mercury-arc lamp or 75W xenon lamp) with power supply 2. There is an XY stage 3 to move the plate 4 in the XY direction over the microscope objective. A Z-axis focus drive 5 moves the objective in the Z direction for focusing. A joystick 6 provides for manual movement  
5 of the stage in the XYZ direction. A high resolution digital camera 7 acquires images from each well or location on the plate. There is a camera power supply 8, an automation controller 9 and a central processing unit 10. The PC 11 provides a display 12 and has associated software. The printer 13 provides for printing of a hard copy record.

10 Figure 2 is a schematic of one embodiment of the microscope assembly 1 of the invention, showing in more detail the XY stage 3, Z-axis focus drive 5, joystick 6, light source 2, and automation controller 9. Cables to the computer 15 and microscope 16, respectively, are provided. In addition, Figure 2 shows a 96 well microtiter plate 17 which is moved on the XY stage 3 in the XY direction. Light from the light source 2  
15 passes through the PC controlled shutter 18 to a motorized filter wheel 19 with excitation filters 20. The light passes into filter cube 25 which has a dichroic mirror 26 and an emission filter 27. Excitation light reflects off the dichroic mirror to the wells in the microtiter plate 17 and fluorescent light 28 passes through the dichroic mirror 26 and the emission filter 27 and to the digital camera 7.

20 Figure 3 shows a schematic drawing of a preferred camera assembly. The digital camera 7, which contains an automatic shutter for exposure control and a power supply 31, receives fluorescent light 28 from the microscope assembly. A digital cable 30 transports digital signals to the computer.

The standard optical configurations described above use microscope optics to directly produce an enlarged image of the specimen on the camera sensor in order to capture a high resolution image of the specimen. This optical system is commonly referred to as 'wide field' microscopy. Those skilled in the art of microscopy will  
5 recognize that a high resolution image of the specimen can be created by a variety of other optical systems, including, but not limited to, standard scanning confocal detection of a focused point or line of illumination scanned over the specimen (Go et al. 1997, *supra*), and multi-photon scanning confocal microscopy (Denk et al., 1990, *supra*), both of which can form images on a CCD detector or by synchronous  
10 digitization of the analog output of a photomultiplier tube.

In screening applications, it is often necessary to use a particular cell line, or primary cell culture, to take advantage of particular features of those cells. Those skilled in the art of cell culture will recognize that some cell lines are contact inhibited, meaning that they will stop growing when they become surrounded by other cells,  
15 while other cell lines will continue to grow under those conditions and the cells will literally pile up, forming many layers. An example of such a cell line is the HEK 293 (ATCC CRL-1573) line. An optical system that can acquire images of single cell layers in multilayer preparations is required for use with cell lines that tend to form layers. The large depth of field of wide field microscopes produces an image that is a  
20 projection through the many layers of cells, making analysis of subcellular spatial distributions extremely difficult in layer-forming cells. Alternatively, the very shallow depth of field that can be achieved on a confocal microscope, (about one micron), allows discrimination of a single cell layer at high resolution, simplifying the

determination of the subcellular spatial distribution. Similarly, confocal imaging is preferable when detection modes such as fluorescence lifetime imaging are required.

The output of a standard confocal imaging attachment for a microscope is a digital image that can be converted to the same format as the images produced by the other cell screening system embodiments described above, and can therefore be processed in exactly the same way as those images. The overall control, acquisition and analysis in this embodiment is essentially the same. The optical configuration of the confocal microscope system, is essentially the same as that described above, except for the illuminator and detectors. Illumination and detection systems required for confocal microscopy have been designed as accessories to be attached to standard microscope optical systems such as that of the present invention (Zeiss, Germany). These alternative optical systems therefore can be easily integrated into the system as described above.

Figure 4 illustrates an alternative embodiment of the invention in which cell arrays are in microwells 40 on a microplate 41, described in co-pending U.S. Application S/N 08/865,341, incorporated by reference herein in its entirety. Typically the microplate is 20 mm by 30 mm as compared to a standard 96 well microtiter plate which is 86 mm by 129 mm. The higher density array of cells on a microplate allows the microplate to be imaged at a low resolution of a few microns per pixel for high throughput and particular locations on the microplate to be imaged at a higher resolution of less than 0.5 microns per pixel. These two resolution modes help to improve the overall throughput of the system.

The microplate chamber 42 serves as a microfluidic delivery system for the addition of compounds to cells. The microplate 41 in the microplate chamber 42 is

placed in an XY microplate reader 43. Digital data is processed as described above. The small size of this microplate system increases throughput, minimizes reagent volume and allows control of the distribution and placement of cells for fast and precise cell-based analysis. Processed data can be displayed on a PC screen 11 and made part  
5 of a bioinformatics data base 44. This data base not only permits storage and retrieval of data obtained through the methods of this invention, but also permits acquisition and storage of external data relating to cells. Figure 5 is a PC display which illustrates the operation of the software.

In an alternative embodiment, a high throughput system (HTS) is directly  
10 coupled with the HCS either on the same platform or on two separate platforms connected electronically (e.g. via a local area network). This embodiment of the invention, referred to as a dual mode optical system, has the advantage of increasing the throughput of a HCS by coupling it with a HTS and thereby requiring slower high resolution data acquisition and analysis only on the small subset of wells that show a  
15 response in the coupled HTS.

High throughput 'whole plate' reader systems are well known in the art and are commonly used as a component of an HTS system used to screen large numbers of compounds (Beggs (1997), *J. of Biomolec. Screening* 2:71-78; Macaffrey et al., (1996) *J. Biomolec. Screening* 1:187-190).

20 In one embodiment of dual mode cell based screening, a two platform architecture in which high throughput acquisition occurs on one platform and high content acquisition occurs on a second platform is provided (Figure 6). Processing occurs on each platform independently, with results passed over a network interface, or a single controller is used to process the data from both platforms.

As illustrated in Figure 6, an exemplified two platform dual mode optical system consists of two light optical instruments, a high throughput platform 60 and a high content platform 65, which read fluorescent signals emitted from cells cultured in microtiter plates or microwell arrays on a microplate, and communicate with each other via an electronic connection 64. The high throughput platform 60 analyzes all the wells in the whole plate either in parallel or rapid serial fashion. Those skilled in the art of screening will recognize that there are a many such commercially available high throughput reader systems that could be integrated into a dual mode cell based screening system (Topcount (Packard Instruments, Meriden, CT); Spectramax, Lumiskan (Molecular Devices, Sunnyvale, CA); Fluoroscan (Labsystems, Beverly, MA)). The high content platform 65, as described above, scans from well to well and acquires and analyzes high resolution image data collected from individual cells within a well.

The HTS software, residing on the system's computer 62, controls the high throughput instrument, and results are displayed on the monitor 61. The HCS software, residing on it's computer system 67, controls the high content instrument hardware 65, optional devices (e.g. plate loader, environmental chamber, fluid dispenser), analyzes digital image data from the plate, displays results on the monitor 66 and manages data measured in an integrated database. The two systems can also share a single computer, in which case all data would be collected, processed and displayed on that computer, without the need for a local area network to transfer the data. Microtiter plates are transferred from the high throughput system to the high content system 63 either manually or by a robotic plate transfer device, as is well known in the art (Beggs (1997), *supra*; Mcaffrey (1996), *supra*).

In a preferred embodiment, the dual mode optical system utilizes a single platform system (Figure 7). It consists of two separate optical modules, an HCS module 203 and an HTS module 209 that can be independently or collectively moved so that only one at a time is used to collect data from the microtiter plate 201. The  
5 microtiter plate 201 is mounted in a motorized X,Y stage so it can be positioned for imaging in either HTS or HCS mode. After collecting and analyzing the HTS image data as described below, the HTS optical module 209 is moved out of the optical path and the HCS optical module 203 is moved into place.

The optical module for HTS 209 consists of a projection lens 214, excitation  
10 wavelength filter 213 and dichroic mirror 210 which are used to illuminate the whole bottom of the plate with a specific wavelength band from a conventional microscope lamp system (not illustrated). The fluorescence emission is collected through the dichroic mirror 210 and emission wavelength filter 211 by a lens 212 which forms an image on the camera 216 with sensor 215.

15 The optical module for HCS 203 consists of a projection lens 208, excitation wavelength filter 207 and dichroic mirror 204 which are used to illuminate the back aperture of the microscope objective 202, and thereby the field of that objective, from a standard microscope illumination system (not shown). The fluorescence emission is collected by the microscope objective 202, passes through the dichroic mirror 204 and  
20 emission wavelength filter 205 and is focused by a tube lens 206 which forms an image on the same camera 216 with sensor 215.

In an alternative embodiment of the present invention, the cell screening system further comprises a fluid delivery device for use with the live cell embodiment of the



method of cell screening (see below). Figure 8 exemplifies a fluid delivery device for use with the system of the invention. It consists of a bank of 12 syringe pumps 701 driven by a single motor drive. Each syringe 702 is sized according to the volume to be delivered to each well, typically between 1 and 100  $\mu$ L. Each syringe is attached via  
5 flexible tubing 703 to a similar bank of connectors which accept standard pipette tips 705. The bank of pipette tips are attached to a drive system so they can be lowered and raised relative to the microtiter plate 706 to deliver fluid to each well. The plate is mounted on an X,Y stage, allowing movement relative to the optical system 707 for data collection purposes. This set-up allows one set of pipette tips, or even a single  
10 pipette tip, to deliver reagent to all the wells on the plate. The bank of syringe pumps can be used to deliver fluid to 12 wells simultaneously, or to fewer wells by removing some of the tips.

In another aspect, the present invention provides a method for analyzing cells comprising providing an array of locations which contain multiple cells wherein the  
15 cells contain one or more fluorescent reporter molecules; scanning multiple cells in each of the locations containing cells to obtain fluorescent signals from the fluorescent reporter molecule in the cells; converting the fluorescent signals into digital data; and utilizing the digital data to determine the distribution, environment or activity of the fluorescent reporter molecule within the cells.

20

### *Cell Arrays*

Screening large numbers of compounds for activity with respect to a particular biological function requires preparing arrays of cells for parallel handling of cells and reagents. Standard 96 well microtiter plates which are 86 mm by 129 mm, with 6mm

diameter wells on a 9mm pitch, are used for compatibility with current automated loading and robotic handling systems. The microplate is typically 20 mm by 30 mm, with cell locations that are 100-200 microns in dimension on a pitch of about 500 microns. Methods for making microplates are described in U.S. Patent Application  
5 Serial No. 08/865,341, incorporated by reference herein in its entirety. Microplates may consist of coplanar layers of materials to which cells adhere, patterned with materials to which cells will not adhere, or etched 3-dimensional surfaces of similarly patterned materials. For the purpose of the following discussion, the terms 'well' and 'microwell' refer to a location in an array of any construction to which cells adhere and  
10 within which the cells are imaged. Microplates may also include fluid delivery channels in the spaces between the wells. The smaller format of a microplate increases the overall efficiency of the system by minimizing the quantities of the reagents, storage and handling during preparation and the overall movement required for the scanning operation. In addition, the whole area of the microplate can be imaged more  
15 efficiently, allowing a second mode of operation for the microplate reader as described later in this document.

#### *Fluorescence Reporter Molecules*

A major component of the new drug discovery paradigm is a continually  
20 growing family of fluorescent and luminescent reagents that are used to measure the temporal and spatial distribution, content, and activity of intracellular ions, metabolites, macromolecules, and organelles. Classes of these reagents include labeling reagents that measure the distribution and amount of molecules in living and fixed cells, environmental indicators to report signal transduction events in time and space, and

fluorescent protein biosensors to measure target molecular activities within living cells. A multiparameter approach that combines several reagents in a single cell is a powerful new tool for drug discovery.

The method of the present invention is based on the high affinity of fluorescent or luminescent molecules for specific cellular components. The affinity for specific components is governed by physical forces such as ionic interactions, covalent bonding (which includes chimeric fusion with protein-based chromophores, fluorophores, and lumiphores), as well as hydrophobic interactions, electrical potential, and, in some cases, simple entrapment within a cellular component. The luminescent probes can be small molecules, labeled macromolecules, or genetically engineered proteins, including, but not limited to green fluorescent protein chimeras.

Those skilled in this art will recognize a wide variety of fluorescent reporter molecules that can be used in the present invention, including, but not limited to, fluorescently labeled biomolecules such as proteins, phospholipids and DNA hybridizing probes. Similarly, fluorescent reagents specifically synthesized with particular chemical properties of binding or association have been used as fluorescent reporter molecules (Barak et al., (1997), *J. Biol. Chem.* 272:27497-27500; Southwick et al., (1990), *Cytometry* 11:418-430; Tsien (1989) in *Methods in Cell Biology*, Vol. 29 Taylor and Wang (eds.), pp. 127-156). Fluorescently labeled antibodies are particularly useful reporter molecules due to their high degree of specificity for attaching to a single molecular target in a mixture of molecules as complex as a cell or tissue.

The luminescent probes can be synthesized within the living cell or can be transported into the cell via several non-mechanical modes including diffusion, facilitated or active transport, signal-sequence-mediated transport, and endocytotic or

pinocytotic uptake. Mechanical bulk loading methods, which are well known in the art, can also be used to load luminescent probes into living cells (Barber et al. (1996), *Neuroscience Letters* 207:17-20; Bright et al. (1996), *Cytometry* 24:226-233; McNeil (1989) in *Methods in Cell Biology*, Vol. 29, Taylor and Wang (eds.), pp. 153-173).

- 5 These methods include electroporation and other mechanical methods such as scrape-loading, bead-loading, impact-loading, syringe-loading, hypertonic and hypotonic loading. Additionally, cells can be genetically engineered to express reporter molecules, such as GFP, coupled to a protein of interest as previously described (Chalfie and Prasher U.S. Patent No. 5,491,084; Cubitt et al. (1995), *Trends in*  
10 *Biochemical Science* 20:448-455).

Once in the cell, the luminescent probes accumulate at their target domain as a result of specific and high affinity interactions with the target domain or other modes of molecular targeting such as signal-sequence-mediated transport. Fluorescently labeled reporter molecules are useful for determining the location, amount and chemical  
15 environment of the reporter. For example, whether the reporter is in a lipophilic membrane environment or in a more aqueous environment can be determined (Giuliano et al. (1995), *Ann. Rev. of Biophysics and Biomolecular Structure* 24:405-434; Giuliano and Taylor (1995), *Methods in Neuroscience* 27:1-16). The pH environment of the reporter can be determined (Bright et al. (1989), *J. Cell Biology* 104:1019-1033;  
20 Giuliano et al. (1987), *Anal. Biochem.* 167:362-371; Thomas et al. (1979), *Biochemistry* 18:2210-2218). It can be determined whether a reporter having a chelating group is bound to an ion, such as  $Ca^{++}$ , or not (Bright et al. (1989), In *Methods in Cell Biology*, Vol. 30, Taylor and Wang (eds.), pp. 157-192; Shimoura et al.

(1988), *J. of Biochemistry* (Tokyo) 251:405-410; Tsien (1989) In *Methods in Cell Biology*, Vol. 30, Taylor and Wang (eds.), pp. 127-156).

Furthermore, certain cell types within an organism may contain components that can be specifically labeled that may not occur in other cell types. For example, epithelial cells often contain polarized membrane components. That is, these cells asymmetrically distribute macromolecules along their plasma membrane. Connective or supporting tissue cells often contain granules in which are trapped molecules specific to that cell type (e.g., heparin, histamine, serotonin, etc.). Most muscular tissue cells contain a sarcoplasmic reticulum, a specialized organelle whose function is to regulate the concentration of calcium ions within the cell cytoplasm. Many nervous tissue cells contain secretory granules and vesicles in which are trapped neurohormones or neurotransmitters. Therefore, fluorescent molecules can be designed to label not only specific components within specific cells, but also specific cells within a population of mixed cell types.

Those skilled in the art will recognize a wide variety of ways to measure fluorescence. For example, some fluorescent reporter molecules exhibit a change in excitation or emission spectra, some exhibit resonance energy transfer where one fluorescent reporter loses fluorescence, while a second gains in fluorescence, some exhibit a loss (quenching) or appearance of fluorescence, while some report rotational movements (Giuliano et al. (1995), *Ann. Rev. of Biophysics and Biomol. Structure* 24:405-434; Giuliano et al. (1995), *Methods in Neuroscience* 27:1-16).

#### *Scanning cell arrays*

Referring to Figure 9, a preferred embodiment is provided to analyze cells that comprises operator-directed parameters being selected based on the assay being

conducted, data acquisition by the cell screening system on the distribution of fluorescent signals within a sample, and interactive data review and analysis. At the start of an automated scan the operator enters information 100 that describes the sample, specifies the filter settings and fluorescent channels to match the biological labels being used and the information sought, and then adjusts the camera settings to match the sample brightness. For flexibility to handle a range of samples, the software allows selection of various parameter settings used to identify nuclei and cytoplasm, and selection of different fluorescent reagents, identification of cells of interest based on morphology or brightness, and cell numbers to be analyzed per well. These parameters are stored in the system's database for easy retrieval for each automated run. The system's interactive cell identification mode simplifies the selection of morphological parameter limits such as the range of size, shape, and intensity of cells to be analyzed. The user specifies which wells of the plate the system will scan and how many fields or how many cells to analyze in each well. Depending on the setup mode selected by the user at step 101, the system either automatically pre-focuses the region of the plate to be scanned using an autofocus procedure to "find focus" of the plate 102 or the user interactively pre-focuses 103 the scanning region by selecting three "tag" points which define the rectangular area to be scanned. A least-squares fit "focal plane model" is then calculated from these tag points to estimate the focus of each well during an automated scan. The focus of each well is estimated by interpolating from the focal plane model during a scan.

During an automated scan, the software dynamically displays the scan status, including the number of cells analyzed, the current well being analyzed, images of each independent wavelength as they are acquired, and the result of the screen for each well

as it is determined. The plate 4 (Figure 1) is scanned in a serpentine style as the software automatically moves the motorized microscope XY stage 3 from well to well and field to field within each well of a 96-well plate. Those skilled in the programming art will recognize how to adapt software for scanning of other microplate formats such as 24, 48, and 384 well plates. The scan pattern of the entire plate as well as the scan pattern of fields within each well are programmed. The system adjusts sample focus with an autofocus procedure 104 (Figure 9) through the Z axis focus drive 5, controls filter selection via a motorized filter wheel 19, and acquires and analyzes images of up to four different colors ("channels" or "wavelengths").

10       The autofocus procedure is called at a user selected frequency, typically for the first field in each well and then once every 4 to 5 fields within each well. The autofocus procedure calculates the starting Z-axis point by interpolating from the pre-calculated plane focal model. Starting a programmable distance above or below this set point, the procedure moves the mechanical Z-axis through a number of different positions, 15       acquires an image at each position, and finds the maximum of a calculated focus score that estimates the contrast of each image. The Z position of the image with the maximum focus score determines the best focus for a particular field. Those skilled in the art will recognize this as a variant of automatic focusing algorithms as described in Harms et al. in *Cytometry* 5 (1984), 236-243, Groen et al. in *Cytometry* 6 (1985), 81-91, 20       and Firestone et al. in *Cytometry* 12 (1991), 195-206.

For image acquisition, the camera's exposure time is separately adjusted for each dye to ensure a high-quality image from each channel. Software procedures can be called, at the user's option, to correct for registration shifts between wavelengths by accounting for linear (X and Y) shifts between wavelengths before making any further

measurements. The electronic shutter 18 is controlled so that sample photo-bleaching is kept to a minimum. Background shading and uneven illumination can be corrected by the software using methods known in the art (Bright et al. (1987), *J. Cell Biol.* 104:1019-1033).

5           In one channel, images are acquired of a primary marker 105 (Figure 9) (typically cell nuclei counterstained with DAPI or PI fluorescent dyes) which are segmented ("identified") using an adaptive thresholding procedure. The adaptive thresholding procedure 106 is used to dynamically select the threshold of an image for separating cells from the background. The staining of cells with fluorescent dyes can  
10       vary to an unknown degree across cells in a microtiter plate sample as well as within images of a field of cells within each well of a microtiter plate. This variation can occur as a result of sample preparation and/or the dynamic nature of cells. A global threshold is calculated for the complete image to separate the cells from background and account for field to field variation. These global adaptive techniques are variants of those  
15       described in the art. (Kittler et al. in *Computer Vision, Graphics, and Image Processing* 30 (1985), 125-147, Ridler et al. in *IEEE Trans. Systems, Man, and Cybernetics* (1978), 630-632.)

          An alternative adaptive thresholding method utilizes local region thresholding in contrast to global image thresholding. Image analysis of local regions leads to better  
20       overall segmentation since staining of cell nuclei (as well as other labeled components) can vary across an image. Using this global/local procedure, a reduced resolution image (reduced in size by a factor of 2 to 4) is first globally segmented (using adaptive thresholding) to find regions of interest in the image. These regions then serve as guides to more fully analyze the same regions at full resolution. A more localized



threshold is then calculated (again using adaptive thresholding) for each region of interest.

The output of the segmentation procedure is a binary image wherein the objects are white and the background is black. This binary image, also called a mask in the art, is used to determine if the field contains objects 107. The mask is labeled with a blob labeling algorithm whereby each object (or blob) has a unique number assigned to it. Morphological features, such as area and shape, of the blobs are used to differentiate blobs likely to be cells from those that are considered artifacts. The user pre-sets the morphological selection criteria by either typing in known cell morphological features or by using the interactive training utility. If objects of interest are found in the field, images are acquired for all other active channels 108, otherwise the stage is advanced to the next field 109 in the current well. Each object of interest is located in the image for further analysis 110. The software determines if the object meets the criteria for a valid cell nucleus 111 by measuring its morphological features (size and shape). For each valid cell, the XYZ stage location is recorded, a small image of the cell is stored, and features are measured 112.

The cell scanning method of the present invention can be used to perform many different assays on cellular samples by applying a number of analytical methods simultaneously to measure features at multiple wavelengths. An example of one such assay provides for the following measurements:

1. The total fluorescent intensity within the cell nucleus for colors 1-4
2. The area of the cell nucleus for color 1 (the primary marker)
3. The shape of the cell nucleus for color 1 is described by three shape features:
  - a) perimeter squared area
  - b) box area ratio
  - c) height width ratio
4. The average fluorescent intensity within the cell nucleus for colors 1-4 (i.e. #1 divided by #2)
5. The total fluorescent intensity of a ring outside the nucleus (see Figure 10) that represents fluorescence of the cell's cytoplasm (cytoplasmic mask) for colors 2-4
6. The area of the cytoplasmic mask
7. The average fluorescent intensity of the cytoplasmic mask for colors 2-4 (i.e. #5 divided by #6)
8. The ratio of the average fluorescent intensity of the cytoplasmic mask to average fluorescent intensity within the cell nucleus for colors 2-4 (i.e. #7 divided by #4)
9. The difference of the average fluorescent intensity of the cytoplasmic mask and the average fluorescent intensity within the cell nucleus for colors 2-4 (i.e. #7 minus #4)
10. The number of fluorescent domains (also call spots, dots, or grains) within the cell nucleus for colors 2-4

Features 1 through 4 are general features of the different cell screening assays of the invention. These steps are commonly used in a variety of image analysis applications and are well known in art (Russ (1992) *The Image Processing Handbook*, CRC Press Inc.; Gonzales et al. (1987), *Digital Image Processing*. Addison-Wesley Publishing Co. pp. 391-448). Features 5-9 have been developed specifically to provide measurements of a cell's fluorescent molecules within the local cytoplasmic region of the cell and the translocation (i.e. movement) of fluorescent molecules from the cytoplasm to the nucleus. These features (steps 5-9) are used for analyzing cells in microplates for the inhibition of nuclear translocation. For example, inhibition of nuclear translocation of transcription factors provides a novel approach to screening intact cells (detailed examples of other types of screens will be provided below). A specific algorithm measures the amount of probe in the nuclear region (feature 4)

versus the local cytoplasmic region (feature 7) of each cell. Quantification of the difference between these two sub-cellular compartments provides a measure of cytoplasm-nuclear translocation (feature 9).

Feature 10 describes a screen used for counting of DNA or RNA probes within  
5 the nuclear region in colors 2-4. For example, probes are commercially available for identifying chromosome-specific DNA sequences (Life Technologies, Gaithersburg, MD; Genosys, Woodlands, TX; Biotechnologies, Inc., Richmond, CA; Bio 101, Inc., Vista, CA) Cells are three-dimensional in nature and when examined at a high magnification under a microscope one probe may be in-focus while another may be  
10 completely out-of-focus. The cell screening method of the present invention provides for detecting three-dimensional probes in nuclei by acquiring images from multiple focal planes. The software moves the Z-axis motor drive 5 (Figure 1) in small steps where the step distance is user selected to account for a wide range of different nuclear diameters. At each of the focal steps, an image is acquired. The maximum gray-level  
15 intensity from each pixel in each image is found and stored in a resulting maximum projection image. The maximum projection image is then used to count the probes. The above algorithm works well in counting probes that are not stacked directly above or below another one. To account for probes stacked on top of each other in the Z-direction, users can select an option to analyze probes in each of the focal planes  
20 acquired. In this mode, the scanning system performs the maximum plane projection algorithm as discussed above, detects probe regions of interest in this image, then further analyzes these regions in all the focal plane images.

After measuring cell features 112 (Figure 9), the system checks if there are any unprocessed objects in the current field 113. If there are any unprocessed objects, it

locates the next object 110 and determines whether it meets the criteria for a valid cell nucleus 111, and measures its features. Once all the objects in the current field are processed, the system determines whether analysis of the current plate is complete 114; if not, it determines the need to find more cells in the current well 115. If the need  
5 exists, the system advances the XYZ stage to the next field within the current well 109 or advances the stage to the next well 116 of the plate.

After a plate scan is complete, images and data can be reviewed with the system's image review, data review, and summary review facilities. All images, data, and settings from a scan are archived in the system's database for later review or for  
10 interfacing with a network information management system. Data can also be exported to other third-party statistical packages to tabulate results and generate other reports. Users can review the images alone of every cell analyzed by the system with an interactive image review procedure 117. The user can review data on a cell-by-cell basis using a combination of interactive graphs, a data spreadsheet of measured  
15 features, and images of all the fluorescence channels of a cell of interest with the interactive cell-by-cell data review procedure 118. Graphical plotting capabilities are provided in which data can be analyzed via interactive graphs such as histograms and scatter plots. Users can review summary data that are accumulated and summarized for all cells within each well of a plate with an interactive well-by-well data review  
20 procedure 119. Hard copies of graphs and images can be printed on a wide range of standard printers.

As a final phase of a complete scan, reports can be generated on one or more statistics of the measured features. Users can generate a graphical report of data summarized on a well-by-well basis for the scanned region of the plate using an

interactive report generation procedure 120. This report includes a summary of the statistics by well in tabular and graphical format and identification information on the sample. The report window allows the operator to enter comments about the scan for later retrieval. Multiple reports can be generated on many statistics and be printed with  
5 the touch of one button. Reports can be previewed for placement and data before being printed.

The above-recited embodiment of the method operates in a single high resolution mode referred to as the high content screening (HCS) mode. The HCS mode provides sufficient spatial resolution within a well (on the order of 1  $\mu\text{m}$ ) to define the  
10 distribution of material within the well, as well as within individual cells in the well. The high degree of information content accessible in that mode, comes at the expense of speed and complexity of the required signal processing.

In an alternative embodiment, a high throughput system (HTS) is directly coupled with the HCS either on the same platform or on two separate platforms  
15 connected electronically (e.g. via a local area network). This embodiment of the invention, referred to as a dual mode optical system, has the advantage of increasing the throughput of an HCS by coupling it with an HTS and thereby requiring slower high resolution data acquisition and analysis only on the small subset of wells that show a response in the coupled HTS.

20 High throughput 'whole plate' reader systems are well known in the art and are commonly used as a component of an HTS system used to screen large numbers of compounds (Beggs et al. (1997), *supra*; McCaffrey et al. (1996), *supra* ). The HTS of the present invention is carried out on the microtiter plate or microwell array by reading

many or all wells in the plate simultaneously with sufficient resolution to make determinations on a well-by-well basis. That is, calculations are made by averaging the total signal output of many or all the cells or the bulk of the material in each well. Wells that exhibit some defined response in the HTS (the 'hits') are flagged by the system. Then on the same microtiter plate or microwell array, each well identified as a hit is measured via HCS as described above. Thus, the dual mode process involves:

1. Rapidly measuring numerous wells of a microtiter plate or microwell array,
2. Interpreting the data to determine the overall activity of fluorescently labeled reporter molecules in the cells on a well-by-well basis to identify "hits" (wells that exhibit a defined response),
3. Imaging numerous cells in each "hit" well, and
4. Interpreting the digital image data to determine the distribution, environment or activity of the fluorescently labeled reporter molecules in the individual cells (i.e. intracellular measurements) and the distribution of the cells to test for specific biological functions

In a preferred embodiment of dual mode processing (Figure 11), at the start of a run 301, the operator enters information 302 that describes the plate and its contents, specifies the filter settings and fluorescent channels to match the biological labels being used, the information sought and the camera settings to match the sample brightness. These parameters are stored in the system's database for easy retrieval for each automated run. The microtiter plate or microwell array is loaded into the cell screening system 303 either manually or automatically by controlling a robotic loading device. An optional environmental chamber 304 is controlled by the system to maintain the temperature, humidity and CO<sub>2</sub> levels in the air surrounding live cells in the microtiter plate or microwell array. An optional fluid delivery device 305 (see Figure 8) is controlled by the system to dispense fluids into the wells during the scan.

High throughput processing 306 is first performed on the microtiter plate or microwell array by acquiring and analyzing the signal from each of the wells in the plate. The processing performed in high throughput mode 307 is illustrated in Figure 12 and described below. Wells that exhibit some selected intensity response in this high throughput mode ("hits") are identified by the system. The system performs a conditional operation 308 that tests for hits. If hits are found, those specific hit wells are further analyzed in high content (micro level) mode 309. The processing performed in high content mode 312 is illustrated in Figure 13. The system then updates 310 the informatics database 311 with results of the measurements on the plate. If there are more plates to be analyzed 313 the system loads the next plate 303; otherwise the analysis of the plates terminates 314.

The following discussion describes the high throughput mode illustrated in Figure 12. The preferred embodiment of the system, the single platform dual mode screening system, will be described. Those skilled in the art will recognize that operationally the dual platform system simply involves moving the plate between two optical systems rather than moving the optics. Once the system has been set up and the plate loaded, the system begins the HTS acquisition and analysis 401. The HTS optical module is selected by controlling a motorized optical positioning device 402 on the dual mode system. In one fluorescence channel, data from a primary marker on the plate is acquired 403 and wells are isolated from the plate background using a masking procedure 404. Images are also acquired in other fluorescence channels being used 405. The region in each image corresponding to each well 406 is measured 407. A feature calculated from the measurements for a particular well is compared with a predefined threshold or intensity response 408, and based on the result the well is either flagged as

a "hit" 409 or not. The locations of the wells flagged as hits are recorded for subsequent high content mode processing. If there are wells remaining to be processed 410 the program loops back 406 until all the wells have been processed 411 and the system exits high throughput mode.

5       Following HTS analysis, the system starts the high content mode processing 501 defined in Figure 13. The system selects the HCS optical module 502 by controlling the motorized positioning system. For each "hit" well identified in high throughput mode, the XY stage location of the well is retrieved from memory or disk and the stage is then moved to the selected stage location 503. The autofocus procedure  
10   504 is called for the first field in each hit well and then once every 5 to 8 fields within each well. In one channel, images are acquired of the primary marker 505 (typically cell nuclei counterstained with DAPI, Hoechst or PI fluorescent dye). The images are then segmented (separated into regions of nuclei and non-nuclei) using an adaptive thresholding procedure 506. The output of the segmentation procedure is a binary mask  
15   wherein the objects are white and the background is black. This binary image, also called a mask in the art, is used to determine if the field contains objects 507. The mask is labeled with a blob labeling algorithm whereby each object (or blob) has a unique number assigned to it. If objects are found in the field, images are acquired for all other active channels 508, otherwise the stage is advanced to the next field 514 in the current  
20   well. Each object is located in the image for further analysis 509. Morphological features, such as area and shape of the objects, are used to select objects likely to be cell nuclei 510, and discard (do no further processing on) those that are considered artifacts. For each valid cell nucleus, the XYZ stage location is recorded, a small image of the cell is stored, and assay specific features are measured 511. The system then



performs multiple tests on the cells by applying several analytical methods to measure features at each of several wavelengths. After measuring the cell features, the systems checks if there are any unprocessed objects in the current field 512. If there are any unprocessed objects, it locates the next object 509 and determines whether it meets the

5 criteria for a valid cell nucleus 510, and measures its features. After processing all the objects in the current field, the system determines whether it needs to find more cells or fields in the current well 513. If it needs to find more cells or fields in the current well it advances the XYZ stage to the next field within the current well 515. Otherwise, the system checks whether it has any remaining hit wells to measure 515. If so, it advances

10 to the next hit well 503 and proceeds through another cycle of acquisition and analysis, otherwise the HCS mode is finished 516.

In an alternative embodiment of the present invention, a method of kinetic live cell screening is provided. The previously described embodiments of the invention are used to characterize the spatial distribution of cellular components at a specific point in

15 time, the time of chemical fixation. As such, these embodiments have limited utility for implementing kinetic based screens, due to the sequential nature of the image acquisition, and the amount of time required to read all the wells on a plate. For example, since a plate can require 30 – 60 minutes to read through all the wells, only very slow kinetic processes can be measured by simply preparing a plate of live cells

20 and then reading through all the wells more than once. Faster kinetic processes can be measured by taking multiple readings of each well before proceeding to the next well, but the elapsed time between the first and last well would be too long, and fast kinetic processes would likely be complete before reaching the last well.

The kinetic live cell extension of the invention enables the design and use of screens in which a biological process is characterized by its kinetics instead of, or in addition to, its spatial characteristics. In many cases, a response in live cells can be measured by adding a reagent to a specific well and making multiple measurements on that well with the appropriate timing. This dynamic live cell embodiment of the invention therefore includes apparatus for fluid delivery to individual wells of the system in order to deliver reagents to each well at a specific time in advance of reading the well. This embodiment thereby allows kinetic measurements to be made with temporal resolution of seconds to minutes on each well of the plate. To improve the overall efficiency of the dynamic live cell system, the acquisition control program is modified to allow repetitive data collection from sub-regions of the plate, allowing the system to read other wells between the time points required for an individual well.

Figure 8 describes an example of a fluid delivery device for use with the live cell embodiment of the invention and is described above. This set-up allows one set of pipette tips 705, or even a single pipette tip, to deliver reagent to all the wells on the plate. The bank of syringe pumps 701 can be used to deliver fluid to 12 wells simultaneously, or to fewer wells by removing some of the tips 705. The temporal resolution of the system can therefore be adjusted, without sacrificing data collection efficiency, by changing the number of tips and the scan pattern as follows. Typically, the data collection and analysis from a single well takes about 5 seconds. Moving from well to well and focusing in a well requires about 5 seconds, so the overall cycle time for a well is about 10 seconds. Therefore, if a single pipette tip is used to deliver fluid to a single well, and data is collected repetitively from that well, measurements can be made with about 5 seconds temporal resolution. If 6 pipette tips are used to deliver

fluids to 6 wells simultaneously, and the system repetitively scans all 6 wells, each scan will require 60 seconds, thereby establishing the temporal resolution. For slower processes which only require data collection every 8 minutes, fluids can be delivered to one half of the plate, by moving the plate during the fluid delivery phase, and then  
5 repetitively scanning that half of the plate. Therefore, by adjusting the size of the sub-region being scanned on the plate, the temporal resolution can be adjusted without having to insert wait times between acquisitions. Because the system is continuously scanning and acquiring data, the overall time to collect a kinetic data set from the plate is then simply the time to perform a single scan of the plate, multiplied by the number  
10 of time points required. Typically, 1 time point before addition of compounds and 2 or 3 time points following addition should be sufficient for screening purposes.

Figure 14 shows the acquisition sequence used for kinetic analysis. The start of processing 801 is configuration of the system, much of which is identical to the standard HCS configuration. In addition, the operator must enter information specific  
15 to the kinetic analysis being performed 802, such as the sub-region size, the number of time points required, and the required time increment. A sub-region is a group of wells that will be scanned repetitively in order to accumulate kinetic data. The size of the sub-region is adjusted so that the system can scan a whole sub-region once during a single time increment, thus minimizing wait times. The optimum sub-region size is  
20 calculated from the setup parameters, and adjusted if necessary by the operator. The system then moves the plate to the first sub-region 803, and to the first well in that sub-region 804 to acquire the prestimulation (time = 0) time points. The acquisition sequence performed in each well is exactly the same as that required for the specific HCS being run in kinetic mode. Figure 15 details a flow chart for that processing. All

of the steps between the start 901 and the return 902 are identical to those described as steps 504 – 514 in Figure 13.

After processing each well in a sub-region, the system checks to see if all the wells in the sub-region have been processed 806 (Figure 14), and cycles through all the wells until the whole region has been processed. The system then moves the plate into position for fluid addition, and controls fluidic system delivery of fluids to the entire sub-region 807. This may require multiple additions for sub-regions which span several rows on the plate, with the system moving the plate on the X,Y stage between additions. Once the fluids have been added, the system moves to the first well in the sub-region 808 to begin acquisition of time points. The data is acquired from each well 809 and as before the system cycles through all the wells in the sub-region 810. After each pass through the sub-region, the system checks whether all the time points have been collected 811 and if not, pauses 813 if necessary 812 to stay synchronized with the requested time increment. Otherwise, the system checks for additional sub-regions on the plate 814 and either moves to the next sub-region 803 or finishes 815. Thus, the kinetic analysis mode comprises operator identification of sub-regions of the microtiter plate or microwells to be screened, based on the kinetic response to be investigated, with data acquisitions within a sub-region prior to data acquisition in subsequent sub-regions.

20

### *Specific Screens*

In another aspect of the present invention, a machine readable storage medium comprising a program containing a set of instructions for causing a cell screening

system to execute procedures for defining the distribution and activity of specific cellular constituents and processes is provided. In a preferred embodiment, the cell screening system comprises a high magnification fluorescence optical system with a stage adapted for holding cells and a means for moving the stage, a digital camera, a light source for receiving and processing the digital data from the digital camera, and a computer means for receiving and processing the digital data from the digital camera. This aspect of the invention comprises programs that instruct the cell screening system to define the distribution and activity of specific cellular constituents and processes, using the luminescent probes, the optical imaging system, and the pattern recognition software of the invention. Preferred embodiments of the machine readable storage medium comprise programs consisting of a set of instructions for causing a cell screening system to execute the procedures set forth in Figures 9, 11, 12, 13, 14, 15, or 28. Another preferred embodiment comprises a program consisting of a set of instructions for causing a cell screening system to execute procedures for detecting the distribution and activity of specific cellular constituents and processes. In most preferred embodiments, the cellular processes include, but are not limited to, nuclear translocation of a protein, cellular hypertrophy, apoptosis, transmembrane receptor internalization, and protease-induced translocation of a protein.

The following examples are intended for purposes of illustration only and should not be construed to limit the scope of the invention, as defined in the claims appended hereto.

The various chemical compounds, reagents, dyes, and antibodies that are referred to in the following Examples are commercially available from such sources as

Sigma Chemical (St. Louis, MO), Molecular Probes (Eugene, OR), Aldrich Chemical Company (Milwaukee, WI), Accurate Chemical Company (Westbury, NY), Jackson Immunolabs, and Clontech (Palo Alto, CA).

5    *Example 1    Automated Screen for Compounds that Induce or Inhibit Nuclear Translocation of a DNA Transcription Factor*

Regulation of transcription of some genes involves activation of a transcription factor in the cytoplasm, resulting in that factor being transported into the nucleus where  
10    it can initiate transcription of a particular gene or genes. This change in transcription factor distribution is the basis of a screen for the cell-based screening system to detect compounds that inhibit or induce transcription of a particular gene or group of genes. A general description of the screen is given followed by a specific example.

The distribution of the transcription factor is determined by labeling the nuclei  
15    with a DNA specific fluorophore like Hoechst 33423 and the transcription factor with a specific fluorescent antibody. After autofocusing on the Hoechst labeled nuclei, an image of the nuclei is acquired in the cell-based screening system at 20x magnification and used to create a mask by one of several optional thresholding methods, as described *supra*. The morphological descriptors of the regions defined by the mask are compared  
20    with the user defined parameters and valid nuclear masks are identified and used with the following algorithm to extract transcription factor distributions. Each valid nuclear mask is eroded to define a slightly smaller nuclear region. The original nuclear mask is then dilated in two steps to define a ring shaped region around the nucleus, which represents a cytoplasmic region. The average antibody fluorescence in each of these  
25    two regions is determined, and the difference between these averages is defined as the

NucCyt Difference. Two examples of determining nuclear translocation are discussed below and illustrated in Figure 10A-J. Figure 10A illustrates an unstimulated cell with its nucleus 200 labeled with a blue fluorophore and a transcription factor in the cytoplasm 201 labeled with a green fluorophore. Figure 10B illustrates the nuclear mask 202 derived by the cell-based screening system. Figure 10C illustrates the cytoplasm 203 of the unstimulated cell imaged at a green wavelength. Figure 10D illustrates the nuclear mask 202 is eroded (reduced) once to define a nuclear sampling region 204 with minimal cytoplasmic distribution. The nucleus boundary 202 is dilated (expanded) several times to form a ring that is 2-3 pixels wide that is used to define the cytoplasmic sampling region 205 for the same cell. Figure 10E further illustrates a side view which shows the nuclear sampling region 204 and the cytoplasmic sampling region 205. Using these two sampling regions, data on nuclear translocation can be automatically analyzed by the cell-based screening system on a cell by cell basis. Figure 10F-J illustrates the strategy for determining nuclear translocation in a stimulated cell. Figure 10F illustrates a stimulated cell with its nucleus 206 labeled with a blue fluorophore and a transcription factor in the cytoplasm 207 labeled with a green fluorophore. The nuclear mask 208 in Figure 10G is derived by the cell based screening system. Figure 10H illustrates the cytoplasm 209 of a stimulated cell imaged at a green wavelength. Figure 10I illustrates the nuclear sampling region 211 and cytoplasmic sampling region 212 of the stimulated cell. Figure 10J further illustrates a side view which shows the nuclear sampling region 211 and the cytoplasmic sampling region 212.

A specific application of this method has been used to validate this method as a screen. A human cell line was plated in 96 well microtiter plates. Some rows of wells

were titrated with agonist, a known inducer of a specific nuclear transcription factor. The cells were then fixed and stained by standard methods with a fluorescein labeled antibody to the transcription factor, and Hoechst 33423. The cell-based screening system was used to acquire and analyze images from this plate and the NucCyt Difference was found to be strongly correlated with the amount of agonist added to the wells as illustrated in Figure 16. In a second experiment, an antagonist to the receptor for the agonist was titrated in the presence of agonist, progressively inhibiting agonist-induced translocation of the transcription factor. The NucCyt Difference was found to strongly correlate with this inhibition of translocation, as illustrated in Figure 17.

Additional experiments have shown that the NucCyt Difference gives consistent results over a wide range of cell densities and reagent concentrations, and can therefore be routinely used to screen compound libraries for specific nuclear translocation activity. Furthermore, the same method can be used with antibodies to other transcription factors, or GFP-transcription factor chimeras, in living and fixed cells, to screen for effects on the regulation of transcription of this and other genes.

Figure 18 is a representative display on a PC screen of data which was obtained in accordance with Example 1. Graph 1 180 plots the difference between the average antibody fluorescence in the nuclear sampling region and cytoplasmic sampling region, NucCyt Difference verses Well #. Graph 2 181 plots the average fluorescence of the antibody in the nuclear sampling region, NP1 average, versus the Well #. Graph 3 182 plots the average antibody fluorescence in the cytoplasmic sampling region, LIP1 average, versus Well #. The software permits displaying data from each cell. For example, Figure 18 shows a screen display 183, the nuclear image 184, and the fluorescent antibody image 185 for cell #26.



NucCyt Difference referred to in graph 1 180 of Figure 18 is the difference between the average cytoplasmic probe (fluorescent reporter molecule) intensity and the average nuclear probe (fluorescent reporter molecule) intensity. NP1 average referred to in graph 2 181 of Figure 18 is the average of cytoplasmic probe (fluorescent reporter molecule) intensity within the nuclear sampling region. L1P1 average referred to in graph 3 182 of Figure 18 is the average probe (fluorescent reporter molecule) intensity within the cytoplasmic sampling region.

10

*Example 2 Automated Screen for Compounds that Induce or Inhibit Hypertrophy in Cardiac Myocytes*

Hypertrophy in cardiac myocytes has been associated with a cascade of alterations in gene expression and can be characterized in cell culture by an alteration in cell size, that is clearly visible in adherent cells growing on a coverslip. A screen is implemented using the following strategy. Myocyte cell line QM7 (Quail muscle clone 7; ATCC CRL-1962) cultured in 96 well plates, can be treated with various compounds and then fixed and labeled with a fluorescent antibody to a cell surface marker and a DNA label like Hoechst. After focusing on the Hoechst labeled nuclei, two images are acquired, one of the Hoechst labeled nuclei and one of the fluorescent antibody. The nuclei are identified by thresholding to create a mask and then comparing the morphological descriptors of the mask with a set of user defined descriptor values. Local regions containing cells are defined around the nuclei. The limits of the cells in those regions are then defined by a local dynamic threshold operation on the same

25

region in the fluorescent antibody image. A sequence of erosions and dilations is used to separate slightly touching cells and a second set of morphological descriptors is used to identify single cells. The area of the individual cells is tabulated in order to define the distribution of cell sizes for comparison with size data from normal and hypertrophic cells. In addition, a second fluorescent antibody to a particular cellular protein, such as one of the major muscle proteins actin or myosin can be included. Images of this second antibody can be acquired and stored with the above images, for later review, to identify anomalies in the distribution of these proteins in hypertrophic cells, or algorithms can be developed to automatically analyze the distributions of the labeled proteins in these images.

*Example 3. Automated Screens for Compounds that Induce or Inhibit Receptor Internalization*

G-protein coupled receptors

G-protein coupled receptors (GPCRs) are a large class of 7 transmembrane-domain cell surface receptors that transmit signals from the extracellular environment to the cell cytoplasm via their interaction with heterotrimeric G-proteins. Activation of these receptors by ligand binding promotes the exchange of GDP for GTP on the associated G-protein, resulting in the dissociation of the G-protein into active  $G\alpha$ -GTP and  $G\beta\gamma$  subunits. The interaction of these subunits with their effectors stimulates a cascade of secondary signals in the cell, such as the production of cyclic AMP (cAMP) and inositol triphosphate ( $IP_3$ ),  $Ca^{++}$  mobilization, and activation of a variety of kinases. A wide range of biological functions are associated with GPCRs, including, but not limited to, smell, taste, perception of light, control of blood pressure,

neurotransmission, endocrine and exocrine function, chemotaxis, exocytosis, embryogenesis and development, cell growth and differentiation, and oncogenesis. GPCRs have therefore become a major potential target for a variety of therapeutic units.

5 GPCRs span the plasma membrane and undergo a relatively slow rate of endocytosis from the cell surface to endosomes in unstimulated cells. Although poorly understood mechanistically, it is known that the presence of agonist increases the rate of receptor internalization dramatically. Once internalized in endosomes, GPCRs may either be recycled back to the plasma membrane or targeted to lysosomes for  
10 degradation. The significance of this sequestration of GPCRs is not yet fully understood. Receptor internalization may play a role in desensitization (loss of functional response) exhibited as a reduction in the ability of the receptor to generate second messenger in the presence of continued stimulation. However, the rate of receptor loss from the surface is usually too slow to account for the rapid rate of  
15 desensitization (Tobin, A.B. et al. (1992) *Mol. Pharmacol.* 42: 1042-1048), and there are examples where the two processes have been shown to be uncoupled (Baumgold, J. et al. (1989) *Neuropharmacology* 28: 1253-1261; Kanbe, S. et al. (1990) *Biochem. Pharmacol.* 40: 1005-1014).

It is likely that endocytosis of receptors may be involved in resensitization (the  
20 reestablishment of the ability of the cell to produce second messenger in response to stimulation). It has been demonstrated for the  $\beta_2$ -adrenergic receptor ( $\beta_2$ -AR) that sequestration deficient mutants as well as receptors treated with agents that block sequestration do not resensitize (Yu, S.S. et al. (1993) *J. Biol. Chem.* 268(1): 337-341; Barak, L.S. et al. (1994) *J. Biol. Chem.* 269(4): 2790-2795). For the  $\beta_2$ -AR, agonist

stimulation results in receptor phosphorylation by protein kinase A and  $\beta_2$ -adrenergic receptor kinase ( $\beta$ -ARK). Subsequently there is uncoupling of the receptor from its G protein as a result of the recruitment and binding of  $\beta$ -arrestin to the receptor, and internalization of the receptor via clathrin-coated pits is initiated. The acidic  
5 endosomal pH favors phosphatase activity, thus enhancing receptor dephosphorylation ( Krueger, K.M. et al. (1997) *J. Biol. Chem.* 272(1): 5-8) and making the receptor available for recycling to the plasma membrane to reassociate with a G-protein. However, resensitization of other receptors, such as the  $M_4$  muscarinic receptors, has been shown to be delayed by endocytosis (Bogatekewitsch, G.S. et al. (1996) *Mol.*  
10 *Pharmacol.* 50: 424-429). Despite the fact that the functional importance of receptor internalization may vary between receptor classes, it remains clear that internalization is a significant step in the pathway of receptor activation and function.

The fundamental importance of cellular processes involving GPCRs makes them a significant target for drug screening. The state of the art for monitoring GPCR-  
15 ligand interactions and receptor internalization is limited to measurements of a single event (*e.g.*, receptor-ligand interaction or receptor loss from the plasma membrane). Current procedures include measurements of binding of labeled ligand (usually radioactively labeled) to whole cells or isolated membrane fractions (WO/97/04214; von Zastrow and Kobilka, *J. Biol. Chem.* 269:18448-18452 (1994); Koch et al., *J. Biol.*  
20 *Chem.* 273:13652-13657 (1998); Tiberi et al., *J. Biol. Chem.* 271:3771-3778 (1996)), the coincident migration of receptors with various markers into subcellular fractions resolved through centrifugation (Seibold et al., *J. Biol. Chem.* 273:7637-7642 (1998); Stefan et al., *Mol. Biol. Cell.* 9:885-899 (1998)), visualization of fluorescently labeled ligand binding to receptors in fixed cells (Tarasova et al., *J. Biol. Chem.* 272:14817-

14824 (1997)), or antibody labeling (either directly or to epitope tags) to identify receptors (von Zastrow and Kobilka, *J. Biol. Chem.* 269:18448-18452 (1994); Segredo et al. (1997) *J. Neurochem.* 68: 2395-2404; Krueger et al. (1997) *J. Biol. Chem.* 272(1): 5-8; Tiberi et al. (1996) *J. Biol. Chem.* 271(7): 3771-3778)). More recently, green  
5 fluorescent protein (GFP)-receptor fusions have been used, which allows visualization of GPCR receptor trafficking in live cells (Kallal, L. et al. (1998) *J. Biol. Chem.* 273(1): 322-328; Tarasova, N.I. et al. (1997) *J. Biol. Chem.* 272(23): 14817-14824). However, this requires confocal imaging to obtain three-dimensional information in order to distinguish whether a receptor has been internalized or has simply moved in the plane  
10 of the plasma membrane. Methods have also been disclosed for the identification of GPCRs, their ligands, and compounds that modulate their activity (WO 98/13353 and WO 97/48820). These methods, however, detect G-protein activation indirectly by ligand binding to the receptor and reporter gene activation. Neither method directly labels the receptor or directly measures the internalization of the receptor as an  
15 indication of receptor activation.

While existing approaches have provided information and a means of measuring receptor function, there remains a need in the art for a method to directly measure ligand-induced receptor internalization with high spatial and temporal resolution as a measure of receptor activation.

20 Therefore, a novel approach to measuring receptor internalization is described here that permits the measurement of receptor internalization in a single step with appropriate automation and throughput. This approach involves fluorescent labeling of the GPCR and the automated measurement of GPCR internalization in stimulated cells. An alternative novel approach described here involves using dual labeled receptors,

comprising a label specific to the amino terminus of the receptor to distinctly label its extracellular domain in addition to a molecular-based chromophore such as GFP or luciferase on the receptor's carboxy terminus to specifically label the intracellular domain. Methods for the construction of such chimeric protein-expressing DNA constructs are well known in the art. (*Molecular Cloning: A Laboratory Manual* (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press), *Gene Expression Technology* (Methods in Enzymology, Vol. 185, edited by D. Goeddel, 1991. Academic Press, San Diego, CA); *PCR Protocols: A Guide to Methods and Applications* (Innis, et al. 1990. Academic Press, San Diego, CA); *Gene Transfer and Expression Protocols*, pp. 109-128, ed. E.J. Murray, The Humana Press Inc., Clifton, N.J.).

A ratio of fluorescence intensity of the two labels is made in unstimulated and stimulated cells. Since the amino terminus of the receptor is only available for labeling while the receptor is inserted in the plasma membrane, the ratio of the two labels in unpermeabilized cells can be used to measure the extent of internalization of the receptor. There is currently no known technology for simultaneously measuring the relative extracellular availability of external and internal domains of membrane receptors.

In a preferred embodiment of the screen for modulators of GPCRs, living cells are obtained from continuous lines of normal or transformed cells, or primary normal or transformed cells obtained directly from animals. The appropriate cells may be transiently or stably transfected with a DNA construct (either plasmid or viral based) that expresses the GPCR of interest fused to a molecular based chromophore at either its amino or carboxy terminus or internally such that the receptor retains function.

Examples of useful molecular-based chromophores include, but are not limited to, GFP and any of its various mutants (Heim and Tsien (1996) *Current Biology* 6: 178-182; Zhang et al. (1996) *Biochem. Biophys. Res. Comm.* 227: 707-711). In addition, any of the luciferases and their mutants could also be used. This would be a novel labeling  
5 technique since the examples of use of this molecular-based chromophore to date have included use as a reporter of gene activity (Yang et al. (1998) *J. Biol. Chem.* 273(17): 10763-10770; Peng et al. (1998) *J. Biol. Chem.* 273(27): 17286-17295; Baldari et al. (1998) *Biologicals* 26(1): 1-5) and construction of biosensors (Campbell and Patel (1983) *Biochem. J.* 216: 185-194; Sala-Newby and Campbell (1992) *FEBS Lett.* 307:  
10 241-244; Jenkins et al. (1990) *Biochem. Soc. Trans.* 18: 463-464) but not as a chimeras for marking a particular protein target. The expression of the GPCR-luminescent protein fusion may be constitutive (driven by any of a variety of promoters, including but not limited to, CMV, SV40, RSV, actin, EF) or inducible (driven by any of a number of inducible promoters including, but not limited to, tetracycline, ecdysone,  
15 steroid-responsive).

Alternatively, the cells are transiently or stably transfected with a DNA construct (either plasmid or viral based) that expresses the GPCR of interest fused to a small peptide or epitope tag. The epitope tag may be fused to the amino or carboxy terminus, or internally such that the receptor remains functional, or, alternatively, the  
20 GPCR may be labeled with two distinct epitope tags, with one being fused to each end of the GPCR. Some examples of epitope tags include, but are not limited to, FLAG (Sigma Chemical, St. Louis, MO), myc (9E10) (Invitrogen, Carlsbad, CA), 6-His (Invitrogen; Novagen, Madison, WI), and HA (Boehringer Mannheim Biochemicals). The expression the GPCR fusion may be constitutive or inducible.

In another embodiment, the cells are transiently or stably transfected with a DNA construct (either plasmid or viral based) that expresses the GPCR of interest fused to an epitope tag at its amino terminus and a molecular based chromophore at its carboxy terminus. Alternatively, the GPCR may be fused to an epitope tag at its carboxyl terminus and a molecular based chromophore at its amino terminus. The expression of the GPCR fusion may be constitutive or inducible.

The appropriate cells are then patterned into arrays for treatment and analysis. These arrays can be multiple well plates containing 96, 384, 1536, or more individual wells. The cells can also be arranged into microarrays of "virtual wells" using the CellChip™ System (U.S. Patent App., Serial No. 08,865,341). These microarrays can be of the same cell type and are treated with a combinatorial of distinct compounds, or alternatively, the microarrays can be a combinatorial of cell types treated with one or more compounds.

Once the chosen cells are patterned into wells or microarrays, they are treated with solutions of drugs or ligands to either inhibit or stimulate receptor internalization. The fluidic delivery system can be manual, robotic, or the microfluidics of the CellChip System (U.S. Patent App., Serial No. 08,865,341). After an appropriate incubation period, the cells are fixed with a chemical crosslinking agent and stained with luminescence-based reagents. These reagents include, but are not limited to, luminescently labeled primary or secondary antibodies that react with the GPCR, the epitope tag, or other cellular antigens determined to correlate with internalization of the GPCR. Luminescent stains, dyes, and other small molecules can also be used to measure the physiological response of the cells to drugs. These reagents are used to



measure the temporal and spatial changes in ions, metabolites, macromolecules, and organelles induced by drugs. Macromolecular-based indicators of cellular physiology can also be used in the assay.

In another embodiment, cells in wells or microarrays are treated with drugs, and the physiological response is measured temporally and spatially within a population of single living cells after an appropriate incubation period. Luminescent stains, dyes, and other small molecules can be used to measure the physiological response of living cells to drugs. Molecular-based chromophores expressed by the cells themselves (such as GFP and its mutants) are particularly suited to live cell measurements. These reagents can be used to measure the temporal and spatial intracellular changes of ions, metabolites, macromolecules, and organelles induced by drugs. Macromolecular-based indicators of cellular physiology can also be used in the assay. These luminescent analogs and biosensors can be used to measure the temporal and spatial changes in the distribution and activity of macromolecules such as protein, DNA, RNA, lipids, and carbohydrates in response to drug treatments.

In another embodiment, fluorescently labeled ligand is used to induce receptor sequestration and the fate of the ligand is following as a parameter of the high-content screen.

In another embodiment, cells may contain more than one distinctly labeled receptor such that different GPCRs can be analyzed in the same cells by using different fluorescence channels to collect those data. Similarly, the wells or microarrays may contain mixed populations of cells, each population containing a different receptor labeled with a spectrally distinct fluorophore. It is possible to measure the effects of drugs on different receptors in a single run by utilizing a cell screening system, such as

the cell screening system of the present invention, that is capable of distinguishing the channels of fluorescence of the different receptors in these examples. In this way one can screen for compounds that affect multiple receptor types or, conversely, for compounds that affect one receptor type and not others.

5           It will be obvious to one skilled in the art that this invention can be applied to any cell surface receptor that undergoes internalization in response to agonist stimulation. Some known examples of GPCRs are the adrenergic receptors; muscarinic acetylcholine receptors; opioid receptors; chemokine receptors; neuropeptide receptors; prostaglandin receptors; parathyroid hormone receptor; cholecystikinin receptor;  
10   secretin receptor; rhodopsin; dopamine receptors; serotonin receptors; odorant receptors; histamine receptors; angiotensin receptors; gastrin receptors; follicle stimulating hormone receptor; luteinizing hormone receptor; metabotropic glutamate receptors; glucagon receptor (a more complete list of known GPCRs and their ligands can be found in Beck-Sickinger, A.G (1996) *Drug Discovery Today* 1(12): 502-513).  
15   This invention is not limited to GPCRs; examples of other receptors to which this invention could be applied include, but are not limited to, growth factor receptors such as PDGF (Heldin et al. (1982) *J. Biol. Chem.* 257(8): 4216-4221; Kapeller et al. (1993) *Mol. Cell. Biol.* 13(10): 6052-6063) and EGF (Zidovetzki et al. (1981) *Proc. Natl. Acad. Sci.* 78(11): 6981-6985; Beguinot et al. (1984) *Proc. Natl. Acad. Sci.* 81(8):  
20   2384-2388; Emlet et al. (1997) *J. Biol. Chem.* 272(7): 4079-4086), the transferrin receptor (Klausner et al. (1983) *J. Biol. Chem.* 258(8): 4715-4724; Ciechanover et al. (1983) *J. Cell. Biochem.* 23(1-4): 107-130), and the insulin receptor (Baldwin et al. (1980) *Proc. Natl. Acad. Sci.* 77(10): 5975-5978; Di Guglielmo et al. (1998) *Mol. Cell.*

Biochem. 182(1-2): 59-63). This invention can also be applied to orphan receptors for which a specific ligand and/or effector is unknown.

The following example is a screen for activation of a G-protein coupled receptor (GPCR) as detected by the translocation of the GPCR from the plasma membrane to a proximal nuclear location. This example illustrates how a high throughput screen can be coupled with a high-content screen in the dual mode System for Cell Based Screening.

Figure 19 illustrates a dual mode screen for activation of a GPCR. Cells carrying a stable chimera of the GPCR with a blue fluorescent protein (BFP) are loaded with the acetoxymethylester form of Fluo-3, a cell permeable calcium indicator (green fluorescence) that is trapped in living cells by the hydrolysis of the esters. They are then deposited into the wells of a microtiter plate 601. The wells are then treated with an array of test compounds using a fluid delivery system, and a short sequence of Fluo-3 images of the whole microtiter plate are acquired and analyzed for wells exhibiting a calcium response (i.e., high throughput mode). The images appear like the illustration of the microtiter plate 601 in Figure 19. A small number of wells, such as wells C4 and E9 in the illustration, would fluoresce more brightly due to the  $\text{Ca}^{++}$  released upon stimulation of the receptors. The locations of wells containing compounds that induced a response 602, would then be transferred to the HCS program and the optics switched for detailed cell by cell analysis of the blue fluorescence for evidence of GPCR translocation to the perinuclear region. The bottom of Figure 19 illustrates the two possible outcomes of the analysis of the high resolution cell data. The camera images a sub-region 604 of the well area 603, producing images of the fluorescent cells 605. In well C4, the uniform distribution of the fluorescence in the cells indicates that the

receptor has not internalized, implying that the  $\text{Ca}^{++}$  response seen was the result of the stimulation of some other signaling system in the cell. The cells in well E9 606 on the other hand, clearly indicate a concentration of the receptor in the perinuclear region clearly indicating the full activation of the receptor. Because only a few hit wells have  
5 to be analyzed with high resolution, the overall throughput of the dual mode system can be quite high, comparable to the high throughput system alone.

*Example 4. High-content screen of ligand-induced parathyroid hormone receptor internalization*

10

*Plasmid construct.* A eukaryotic expression plasmid containing the coding sequence for a humanized GFP mutant (pEGFP-N<sub>2</sub>, CLONTECH, Palo Alto, CA) was used to create a GFP-human parathyroid hormone receptor (PTHr, GenBank #L04308) chimera.

15

*Cell preparation.* The plasmid construct was used to transfect a human embryonic kidney cell line (HEK 293) (ATCC NO.CRL-1573). Clonal lines stably expressing the GFP-PTHr chimera were established by antibiotic selection with the neomycin analog Geneticin (0.5 mg/ml; Life Technologies, Gaithersburg, MD). Cells are prepared and plated in DMEM/F12 medium (Life Technologies) containing 25mM HEPES buffer  
20 (no sodium bicarbonate), 10% fetal calf serum (FCS), penicillin/streptomycin (PS), and 2mM L-glutamine. Cells are plated at a density of  $4 \times 10^4$  per well in a 96-well microtiter plate in a volume of 200ul per well. Cells are allowed to settle for approximately 30 minutes at room temperature and the plate is then placed in a 37°C humidified air incubator.

25

*Parathyroid hormone induction of GFP-PTHr internalization.* A 100uM stock of bovine parathyroid hormone (PTH), amino acids 1-34 (Bachem, King of Prussia, PA), is prepared using acidified water (pH 4-4.5). To induce internalization of the GFP-PTHr chimera, cells are stimulated by the addition of 50ul of 500 nM PTH to each well (diluted in DMEM/F12, 10% FCS, PS, 2mM L-glutamine). This volume is added to the 200ul of medium already in the well, yielding a final concentration of 100nM PTH. The plate is incubated at room temperature for two hours while covered with aluminum foil to protect the fluorophore from light. Following the two hour PTH stimulation, the media is decanted from the plate and the cells are fixed and the nuclei stained by the addition of 200ul of Hank's Balanced Salt Solution (HBSS) containing 3.7% formalin (Sigma) and 1ug/ml Hoechst 33342 (Molecular Probes, Eugene, Oregon). After a 10 minute incubation at room temperature, the solution is decanted from the plate, cells are washed by the addition of 200ul/well HBSS, and the plates are analyzed/stored with fresh HBSS (200ul/well).

15

*Image acquisition and analysis.* (See Figure 26 for overview) After autofocusing 101 (Figure 27) on the Hoechst-labeled nuclei, an image of the nuclei 102 is acquired at 20x magnification. The nucleus image is segmented by thresholding 103, using a threshold value selected by the user or obtained by one of two other methods from which the user can select (isodata algorithm or peak interpolation). The total area in pixels of all the nuclei in the image is then computed as a single sum 104. An image of the GFP fluorescence is then acquired at 20x magnification 105. (Figure 26) The area of the plate imaged in this way is called a field.

Large artifacts are removed from the GFP image as follows 106. (Figure 26)  
The image is thresholded at a user-selected intensity value which is higher than the  
threshold used to detect valid objects later. All objects detected in the resulting image  
are labeled and their size (number of pixels) is measured. Any objects greater than a  
5 user-specified size are treated as artifacts. All such objects are copied and pasted onto a  
new blank image, the artifact image. The artifact image is dilated slightly to be sure  
that the artifacts will be completely deleted. The artifact image is then subtracted from  
the original GFP image, yielding the intermediate image.

To remove variations in the background fluorescence, the intermediate image is  
10 subjected to a top hat transform 107. (Figure 26) This transform consists of  
(a) grayscale erosion (replacement of each pixel value by the minimum value in its  
neighborhood) (b) a grayscale dilation (replacement of each pixel value by the  
maximum value in its neighborhood) with the same size neighborhood as (a),  
producing a background image, and (c) subtraction of the resulting background image  
15 from the original input image to produce the object image, which contains small bright  
spots. The size of the neighborhood used for steps (a) and (b) above is selected to be  
slightly larger than all the objects of interest in the image. As a result, all such objects  
are absent from the background image after the erosion (a) and dilation (b). However,  
gradual variations in the background of the original image are retained in the  
20 background image. Therefore, the subtraction step (c) removes these variations in the  
background from the object image.

The object image is processed to determine which bright spots represent the  
internalized receptor in stimulated cells. This process uses a brightness threshold and a  
minimum size set by the user. The object image is thresholded at the brightness

threshold to create the binary object mask 108. (Figure 26) The objects in the binary object mask are labeled and their sizes are measured in pixels. Those objects that meet or exceed the minimum size are valid spots 109; (Figure 28) the rest are ignored.

The following measurements are then determined for each valid spot. (Figure 28) The count of spots in the field is incremented 110. The number of pixels was previously counted. For each valid spot, the region with its label is extracted from the binary object mask to create the single-spot binary mask. The single-spot binary mask is applied to the original object image to get the grayscale spot image of the respective spot. The intensities of the pixels in the grayscale spot image are summed to get the aggregate intensity of the spot 111. Once all the spots have been processed, the sum of all of the areas of the valid spots are summed to get the aggregate spot area for the field 112. The aggregate intensity of the spots is totaled to get the aggregate spot intensity of the field. There are several statistics to choose from for the final score for the field (or well): (a) the number of valid spots; (b) the aggregate area of the valid spots; (c) the aggregate intensity of the valid spots; (d) the aggregate intensity of the valid spots divided by the total area of the nuclei. When more than one field is analyzed within each well, the values for all the fields of the well are averaged together to get an aggregate statistic for the well 113. (Figure 26)

The following examples of determining receptor internalization using the above techniques illustrate the differences found between treated and untreated cells. The nuclei of unstimulated cells are labeled with the DNA-specific Hoechst stain and imaged with a near-UV fluorescence filter set. The same cells are imaged with a blue fluorescence filter set which shows the distribution of the GFP fluorescence. The nuclear mask is derived by applying a threshold to the nucleus-labeled image, and the

background image is derived by the grayscale erosion and dilation of the GFP image, showing the variations in the background intensity. The object image is then derived by subtracting the background image from the GFP image, resulting in faint spots. The object mask is then derived by applying the threshold to the object image. Some faint  
5 spots are eliminated by the thresholding. Some others have fewer pixels over the threshold than the requirement for a valid spot. As a result, very few valid spots are found in the image of unstimulated cells. The spot count, aggregate spot areas, and aggregate spot brightness all have low values.

In a second example, the nuclei of stimulated cells are labeled with the  
10 DNA-specific Hoechst stain and imaged as in the preceding example. The nuclear mask is derived by the automated thresholding method, and the background image is derived by the grayscale erosion and dilation of the GFP image, showing the variations in the background intensity. The object image is derived by subtracting the background image from the GFP image, resulting in bright spots. The object mask is derived by  
15 applying the threshold to the object image. Many spots are seen in the object mask, and many of those spots have enough pixels over the threshold to meet the requirement for valid spots. The spot count, aggregate spot areas, and aggregate spot brightness all have high values. Results from experiments like these examples were shown previously in Figure 25.

20 **Figure 29** shows a representative display of a PC screen showing data which was obtained by the methods described in the above examples. Each data point represents the Spot Count of a single well of the plate, calculated by summing together the Spot Counts of the fields of the well. The graph 300 shows individual curves, each representing a single row of the 96 well plate. The leftmost six points of each curve



represent the Spot Counts of untreated wells, while the rightmost six points represent treated wells. The Spot Count feature ("obj count" in illustration) can be selected using the list 302. The numerical values for all the rows are shown in spreadsheet format 303. The graph 300 and spreadsheet 303 can be printed, and the spreadsheet can be  
5 exported in a comma-separated format for input into a spreadsheet program such as Microsoft Excel <sup>TM</sup>.

Alternatively, the data can be displayed on a field by field basis (Figure 30). Each graph at the top 304, 305, and 306 can be set to plot any one of the computed statistics (averaged over the fields of the well) vs. the well number. The spreadsheet  
10 307 shows the numerical data computed on a field by field basis. Selection of a line from the spreadsheet causes display of the corresponding Hoechst 308 and GFP 309 images to be displayed. The spreadsheet 305 can be printed or exported in an ASCII file format for input into a spreadsheet program such as Microsoft Excel <sup>TM</sup>.

The graph 304 shows the Spot Count vs. the well number. The Spot Count is  
15 the number of valid spots detected in the input GFP images. The invention provides a computer means for converting the digital signal from the camera into this parameter and for plotting the parameter vs. the well number.

The graph 305 shows the aggregate spot area ("total spot area" in illustration) vs. the well number. The aggregate spot area is the summed areas of all valid spots  
20 detected in the input GFP images. The invention provides a computer means for converting the digital signal from the camera into this parameter and for plotting this parameter vs. the well number.

The graph 306 shows the normalized spot intensity ratio ("Spot Inten Ratio x 100" in illustration) vs. the well number. The normalized spot intensity ratio is the

summed intensities of all the pixels in the valid spots detected in the input GFP images, divided by the summed number of pixels in the nucleus masks in the corresponding Hoechst image. The invention provides a computer means for converting the digital signal from the camera into this parameter and for plotting the parameter vs. the well  
5 number.

Figure 25 is a graphical representation of data from validation runs of the PTHR internalization screen. The figure illustrates that the data for min. ("minimum response" = unstimulated) and max. ("maximum response" = stimulated) are consistent between different plates (the differences are not statistically significant), giving c.o.v.'s  
10 (coefficients of variance) within a consistent and acceptable range.

In a specific example of a high-content screen, four fields were acquired in each well. The Spot Count was summed across the fields of a well, and averaged among the similarly treated wells. The untreated half of the plate had a Spot Count of  $69.3 \pm 17.7$  (mean  $\pm$  Standard Deviation) times the untreated half of the plate, giving a Coefficient  
15 of Variation (COV, the Standard Deviation divided by the mean) of 26%. The values from the fields of the treated half of the plate had a Spot Count of  $404.2 \pm 41.2$ , giving a COV of (10%). The mean Spot Count of the treated half was 5.83 times the mean Spot Count of the untreated half.

#### 20 Example 5 Kinetic High Content Screen

Simply detecting the endpoint as internalized or not, may not be sufficient for defining the potency of a compound as a receptor agonist or antagonist. In another embodiment, the cells are treated with drug and data are collected at various timepoints following drug treatment in order to quantitate the kinetics of receptor internalization.

These kinetic assays can be done on live cells as described above, or different wells of cells can be fixed at each of the various timepoints of interest following drug treatment. In either case, cells can be labeled using the reagents and methods described above. Such kinetic measurements would provide information not only about potency during  
5 the time course of measurement, but would also allow extrapolation of the data to much longer time periods.

In a preferred embodiment, kinetic measurements are first made in one channel of fluorescence in a high-throughput or ultra-high-throughput mode for a cellular response associated with receptor internalization. This response is less receptor  
10 specific than the internalization process itself and may include, but is not limited to, changes in  $\text{Ca}^{2+}$ , cAMP, or  $\text{IP}_3$  concentrations, or activation of any of a variety of kinases. Wells exhibiting the desired output from this parameter are then analyzed in the HCS mode for highly detailed temporal and spatial information on a cell-by-cell basis.

15 The luminescence signals of live or fixed cells are analyzed using a cell scanning system, such as the cell scanning system of the present invention.

*Example 6. Inserted Sequences and their Ligands for High-Content Screens  
Incorporating Dual-Labeled Receptors*

20 In another embodiment, a membrane receptor is modified to contain specific peptide sequences fused to each end in order to distinctly label the extracellular and intracellular domains. A ratio of fluorescence intensity of the two labels is made in unstimulated and stimulated cells; since the amino terminus of the receptor is only available for labeling while the receptor is inserted in the plasma membrane, the ratio  
25 of the two labels in unpermeabilized cells can be used to measure the extent of

internalization of the receptor. There is currently no known technology for simultaneously measuring the relative extracellular availability of external and internal domains of membrane receptors.

Appropriate cells are transiently or stably transfected with a DNA construct  
5 (either plasmid or viral based) that expresses the GPCR of interest fused to an epitope tag at its amino terminus and a molecular based chromophore at its carboxy terminus. Alternatively, the GPCR may be fused to an epitope tag at its carboxyl terminus and a molecular based chromophore at its amino terminus. The expression of the GPCR fusion may be constitutive or inducible.

10 Some examples of epitope tags include, but are not limited to, FLAG (Sigma Chemical, St. Louis, MO), myc (9E10) (Invitrogen, Carlsbad, CA), 6-His (Invitrogen; Novagen, Madison, WI), and HA (Boehringer Mannheim Biochemicals). The expression of the GPCR fusion may be constitutive or inducible.

Examples of useful molecular-based chromophores include, but are not limited  
15 to, GFP and any of its various mutants (Heim and Tsien (1996) *Current Biology* 6: 178-182; Zhang et al. (1996) *Biochem. Biophys. Res. Comm.* 227: 707-711). In addition, any of the luciferases and their mutants could also be used. The use of a luciferase as part of a chimeric target protein comprises a novel labeling technique since the examples of use of this molecular-based chromophore to date have included use as a  
20 reporter of gene activity (Yang et al. (1998) *J. Biol. Chem.* 273(17): 10763-10770; Peng et al. (1998) *J. Biol. Chem.* 273(27): 17286-17295; Baldari et al. (1998) *Biologicals* 26(1): 1-5)) and construction of biosensors (Campbell and Patel (1983) *Biochem. J.* 216: 185-194; Sala-Newby and Campbell (1992) *FEBS Lett.* 307: 241-244; Jenkins et al. (1990) *Biochem. Soc. Trans.* 18: 463-464) but not as a chimera for

marking a particular protein target. Expression of the membrane protein-luminescent protein fusion may be constitutive (driven by any of a variety of promoters, including but not limited to, CMV, SV40, RSV, actin, EF) or inducible (driven by any of a number of inducible promoters including, but not limited to, tetracycline, ecdysone,  
5 steroid-responsive).

Alternatively, cells are transiently or stably transfected with a DNA construct (either plasmid or viral based) that expresses the membrane protein of interest fused to two distinct epitope tags, with one being fused to each end of the membrane protein.

The external availability of the inserted sequences depends on the  
10 internalization state of the receptor. That is, the ratio of the external availability of the inserted sequences provides a direct measure of the magnitude of receptor internalization. This is a high-content screen incorporating dual-labeled receptors. The external availability of the inserted sequences can be measured using a single approach or a combination of several approaches:

15

1. One or more of the inserted sequences can be epitopes for specific antibodies.

Antibody binding to the epitope can be measured using histochemical, radioactive, or fluorescence methods. Possible epitopes include, but are not limited to, those shown in Table I.

20

#### **TABLE I. PEPTIDE EPITOPES AND THEIR CORRESPONDING ANTIBODIES**

ANTIBODY	EPITOPE	SOURCE
FLAG	MDYKDDDDK	Sigma
Myc	EQKLISEEDL	Invitrogen, Boehringer-Mannheim Biochemical(BMB)
6-His	HHHHHH	Invitrogen, BMB, Berkley Antibody Company (BAbCO)
AU1	DTYRYI	BAbCO
AU5	TDFYLK	BAbCO
Glu-Glu	EEEEYMPME	BAbCO
HA	YPYDVPDYA	BMB, BAbCO
IRS	NPDSEIARYIRS	BAbCO
KT-3	KPPTPPPEPET	BAbCO
Protein C	EDQVDPRLIDG K	BMB
VSV-G	YTDIEMNRLGK	BMB
HSV	QPELAPEDPED	Novagen
T7	MASMTGGGQQ MG	Novagen
V5	GKPIPNNLLGLD ST	Invitrogen
Xpress™	DLYDDDDK	Invitrogen

2. The inserted sequences can code for fluorescent proteins. Besides the natural fluorophores of trp, tyr, and phe that exist in many proteins, other fluorescent protein sequences can be inserted. The GFP sequence or one of its mutant variants can be inserted into the sequence coding for the receptor. Sequences coding for luciferase and its mutant variants can also be inserted. Any peptide sequence that codes for or interacts with a fluorophore can be used in this method. The inserted sequences can be structured to express fluorescent proteins with different fluorescent properties such that fluorescent signals from each can be measured independently.
3. The inserted sequences can code for peptides that bind small (<1000 M<sub>r</sub>) ligands with high affinity ( $K_d < 10^{-9}$ ) and specificity. These small molecules then form a

tight bridge to other molecules or macromolecules that can be luminescently or radioactively labeled. The inserted sequences can be structured to bind different bridging molecules that bind distinctly labeled molecules or macromolecules such that signals from each can be measured independently. For example, the peptide  
5 sequence -HHHHHH- will bind a metal ion (*e.g.*,  $\text{Ni}^{2+}$ ,  $\text{Cu}^{2+}$ , *etc.*) that will form a tight bridge with a polydentate acetic acid moiety (*e.g.*, nitriloacetic acid). The acid moiety can be covalently linked to molecules that are luminescent, radioactive, or otherwise light absorbing. These molecules can be luminescent dyes or macromolecules such as proteins that contain a luminescent or radioactive label.  
10 Other examples of inserted peptide sequences are such that they have a high affinity for other small molecules that include steroid hormones, vitamins, and carbohydrates that form a tight bridge to other molecules or macromolecules that can be luminescently or radioactively labeled.

15

*Example 7. A Generalized Dual-labeled Receptor Internalization High-Content*

*Screen*

A modified G-protein coupled receptor (GPCR of known function or orphan) is  
20 transfected into human epithelial kidney cells (HEK 293) where its localization provides a measure of internalization from the plasma membrane. The modified GPCR contains an epitope (for example, FLAG) label at the N-terminus (extracellular) and a GFP-molecule at the C-terminus (intracellular). To measure GPCR internalization after ligand treatment, cells are fixed and labeled with Hoechst 33342 (a DNA-binding

fluorescent dye) and a distinct luminescently labeled antibody against the epitope tag. A cell screening system, such as the cell screening system of the present invention, using ratio imaging, is used to calculate the internalization of the GPCR due to the loss of GPCR-epitope from the external side of the plasma membrane and an increase in  
5 GFP-only-labeled receptor within the cell. This approach to measuring ligand-induced receptor internalization is independent of the internalization mechanism so it is therefore applicable to a wide range of receptors of both known and unknown function.

*Example 8 High-content screen of human glucocorticoid receptor translocation*

10 One class of HCS involves the drug-induced dynamic redistribution of intracellular constituents. The human glucocorticoid receptor (hGR), a single "sensor" in the complex environmental response machinery of the cell, binds steroid molecules that have diffused into the cell. The ligand-receptor complex translocates to the nucleus where transcriptional activation occurs (Htun et al., *Proc. Natl. Acad. Sci.*  
15 93:4845, 1996).

In general, hormone receptors are excellent drug targets because their activity lies at the apex of key intracellular signaling pathways. Therefore, a high-content screen of hGR translocation has distinct advantage over *in vitro* ligand-receptor binding assays. The availability of up to two more channels of fluorescence in the cell  
20 screening system of the present invention permits the screen to contain two additional parameters in parallel, such as other receptors, other distinct targets or other cellular processes.

**Plasmid construct.** A eukaryotic expression plasmid containing a coding sequence for a green fluorescent protein – human glucocorticoid receptor (GFP-hGR)



chimera was prepared using GFP mutants (Palm et al., *Nat. Struct. Biol.* 4:361 (1997). The construct was used to transfect a human cervical carcinoma cell line (HeLa).

**Cell preparation and transfection.** HeLa cells (ATCC CCL-2) were trypsinized and plated using DMEM containing 5% charcoal/dextran-treated fetal bovine serum (FBS) (HyClone) and 1% penicillin-streptomycin (C-DMEM) 12-24 hours prior to transfection and incubated at 37°C and 5% CO<sub>2</sub>. Transfections were performed by calcium phosphate co-precipitation (Graham and Van der Eb, *Virology* 52:456, 1973; Sambrook et al., (1989). *Molecular Cloning: A Laboratory Manual*, Second ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989) or with Lipofectamine (Life Technologies, Gaithersburg, MD). For the calcium phosphate transfections, the medium was replaced, prior to transfection, with DMEM containing 5% charcoal/dextran-treated FBS. Cells were incubated with the calcium phosphate-DNA precipitate for 4-5 hours at 37°C and 5% CO<sub>2</sub>, washed 3-4 times with DMEM to remove the precipitate, followed by the addition of C-DMEM.

Lipofectamine transfections were performed in serum-free DMEM without antibiotics according to the manufacturer's instructions (Life Technologies, Gaithersburg, MD). Following a 2-3 hour incubation with the DNA-liposome complexes, the medium was removed and replaced with C-DMEM. All transfected cells in 96-well microtiter plates were incubated at 33°C and 5% CO<sub>2</sub> for 24-48 hours prior to drug treatment. Experiments were performed with the receptor expressed transiently in HeLa cells.

**Dexamethasone induction of GFP-hGR translocation.** To obtain receptor-ligand translocation kinetic data, nuclei of transfected cells were first labeled with 5 µg/ml Hoechst 33342 (Molecular Probes) in C-DMEM for 20 minutes at 33°C and 5%

CO<sub>2</sub>. Cells were washed once in Hank's Balanced Salt Solution (HBSS) followed by the addition of 100 nM dexamethasone in HBSS with 1% charcoal/dextran-treated FBS. To obtain fixed time point dexamethasone titration data, transfected HeLa cells were first washed with DMEM and then incubated at 33°C and 5% CO<sub>2</sub> for 1 h in the presence of 0 – 1000 nM dexamethasone in DMEM containing 1% charcoal/dextran-treated FBS. Cells were analyzed live or they were rinsed with HBSS, fixed for 15 min with 3.7% formaldehyde in HBSS, stained with Hoechst 33342, and washed before analysis. The intracellular GFP-hGR fluorescence signal was not diminished by this fixation procedure.

10        *Image acquisition and analysis.* Kinetic data were collected by acquiring fluorescence image pairs (GFP-hGR and Hoechst 33342-labeled nuclei) from fields of living cells at 1 min intervals for 30 min after the addition of dexamethasone. Likewise, image pairs were obtained from each well of the fixed time point screening plates 1 h after the addition of dexamethasone. In both cases, the image pairs obtained  
15        at each time point were used to define nuclear and cytoplasmic regions in each cell. Translocation of GFP-hGR was calculated by dividing the integrated fluorescence intensity of GFP-hGR in the nucleus by the integrated fluorescence intensity of the chimera in the cytoplasm or as a nuclear-cytoplasmic difference of GFP fluorescence. In the fixed time point screen this translocation ratio was calculated from data obtained  
20        from at least 200 cells at each concentration of dexamethasone tested. Drug-induced translocation of GFP-hGR from the cytoplasm to the nucleus was therefore correlated with an increase in the translocation ratio.

*Results.* Figure 20 schematically displays the drug-induced cytoplasm 253 to nucleus 252 translocation of the human glucocorticoid receptor. The upper pair of

schematic diagrams depicts the localization of GFP-hGR within the cell before 250 (A) and after 251 (B) stimulation with dexamethasone. Under these experimental conditions, the drug induces a large portion of the cytoplasmic GFP-hGR to translocate into the nucleus. This redistribution is quantified by determining the integrated intensities ratio of the cytoplasmic and nuclear fluorescence in treated 255 and untreated 254 cells. The lower pair of fluorescence micrographs show the dynamic redistribution of GFP-hGR in a single cell, before 254 and after 255 treatment. The HCS is performed on wells containing hundreds to thousands of transfected cells and the translocation is quantified for each cell in the field exhibiting GFP fluorescence.

10 Although the use of a stably transfected cell line would yield the most consistently labeled cells, the heterogeneous levels of GFP-hGR expression induced by transient transfection did not interfere with analysis by the cell screening system of the present invention.

To execute the screen, the cell screening system scans each well of the plate, images a population of cells in each, and analyzes cells individually. Here, two channels of fluorescence are used to define the cytoplasmic and nuclear distribution of the GFP-hGR within each cell. Depicted in Figure 21 is the graphical user interface of the cell screening system near the end of a GFP-hGR screen. The user interface depicts the parallel data collection and analysis capability of the system. The windows labeled

20 "Nucleus" 261 and "GFP-hGR" 262 show the pair of fluorescence images being obtained and analyzed in a single field. The window labeled "Color Overlay" 260 is formed by pseudocoloring the above images and merging them so the user can immediately identify cellular changes. Within the "Stored Object Regions" window 265, an image containing each analyzed cell and its neighbors is presented as it is

archived. Furthermore, as the HCS data are being collected, they are analyzed, in this case for GFP-hGR translocation, and translated into an immediate "hit" response. The 96 well plate depicted in the lower window of the screen 267 shows which wells have met a set of user-defined screening criteria. For example, a white-colored well 269 indicates that the drug-induced translocation has exceeded a predetermined threshold value of 50%. On the other hand, a black-colored well 270 indicates that the drug being tested induced less than 10% translocation. Gray-colored wells 268 indicate "hits" where the translocation value fell between 10% and 50%. Row "E" on the 96 well plate being analyzed 266 shows a titration with a drug known to activate GFP-hGR translocation, dexamethasone. This example screen used only two fluorescence channels. Two additional channels (Channels 3 263 and 4 264) are available for parallel analysis of other specific targets, cell processes, or cytotoxicity to create multiple parameter screens.

There is a link between the image database and the information database that is a powerful tool during the validation process of new screens. At the completion of a screen, the user has total access to image and calculated data (Figure 22). The comprehensive data analysis package of the cell screening system allows the user to examine HCS data at multiple levels. Images 276 and detailed data in a spread sheet 279 for individual cells can be viewed separately, or summary data can be plotted. For example, the calculated results of a single parameter for each cell in a 96 well plate are shown in the panel labeled Graph 1 275. By selecting a single point in the graph, the user can display the entire data set for a particular cell that is recalled from an existing database. Shown here are the image pair 276 and detailed fluorescence and morphometric data from a single cell (Cell #118, gray line 277). The large graphical

insert 278 shows the results of dexamethasone concentration on the translocation of GFP-hGR. Each point is the average of data from at least 200 cells. The calculated EC<sub>50</sub> for dexamethasone in this assay is 2 nM.

A powerful aspect of HCS with the cell screening system is the capability of kinetic measurements using multicolor fluorescence and morphometric parameters in living cells. Temporal and spatial measurements can be made on single cells within a population of cells in a field. Figure 23 shows kinetic data for the dexamethasone-induced translocation of GFP-hGR in several cells within a single field. Human HeLa cells transfected with GFP-hGR were treated with 100 nM dexamethasone and the translocation of GFP-hGR was measured over time in a population of single cells. The graph shows the response of transfected cells 285, 286, 287, and 288 and non-transfected cells 289. These data also illustrate the ability to analyze cells with different expression levels.

*Example 9 High-content screen of drug-induced apoptosis*

Apoptosis is a complex cellular program that involves myriad molecular events and pathways. To understand the mechanisms of drug action on this process, it is essential to measure as many of these events within cells as possible with temporal and spatial resolution. Therefore, an apoptosis screen that requires little cell sample preparation yet provides an automated readout of several apoptosis-related parameters would be ideal. A cell-based assay designed for the cell screening system has been used to simultaneously quantify several of the morphological, organellar, and macromolecular hallmarks of paclitaxel-induced apoptosis.

**Cell preparation.** The cells chosen for this study were mouse connective tissue fibroblasts (L-929; ATCC CCL-1) and a highly invasive glioblastoma cell line (SNB-19; ATCC CRL-2219) (Welch et al., *In Vitro Cell. Dev. Biol.* 31:610, 1995). The day before treatment with an apoptosis inducing drug, 3500 cells were placed into each well of a 96-well plate and incubated overnight at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. The following day, the culture medium was removed from each well and replaced with fresh medium containing various concentrations of paclitaxel (0 – 50 µM) from a 20 mM stock made in DMSO. The maximal concentration of DMSO used in these experiments was 0.25%. The cells were then incubated for 26 h as above. At the end of the paclitaxel treatment period, each well received fresh medium containing 750 nM MitoTracker Red (Molecular Probes; Eugene, OR) and 3 µg/ml Hoechst 33342 DNA-binding dye (Molecular Probes) and was incubated as above for 20 min. Each well on the plate was then washed with HBSS and fixed with 3.7% formaldehyde in HBSS for 15 min at room temperature. The formaldehyde was washed out with HBSS and the cells were permeabilized for 90 s with 0.5% (v/v) Triton X-100, washed with HBSS, incubated with 2 U ml<sup>-1</sup> Bodipy FL phalloidin (Molecular Probes) for 30 min, and washed with HBSS. The wells on the plate were then filled with 200 µl HBSS, sealed, and the plate stored at 4°C if necessary. The fluorescence signals from plates stored this way were stable for at least two weeks after preparation. As in the nuclear translocation assay, fluorescence reagents can be designed to convert this assay into a live cell high-content screen.

**Image acquisition and analysis on the ArrayScan System.** The fluorescence intensity of intracellular MitoTracker Red, Hoechst 33342, and Bodipy FL phalloidin was measured with the cell screening system as described *supra*. Morphometric data

from each pair of images obtained from each well was also obtained to detect each object in the image field (e.g., cells and nuclei), and to calculate its size, shape, and integrated intensity.

**Calculations and output.** A total of 50-250 cells were measured per image field. For each field of cells, the following calculations were performed: (1) The average nuclear area ( $\mu\text{m}^2$ ) was calculated by dividing the total nuclear area in a field by the number of nuclei detected. (2) The average nuclear perimeter ( $\mu\text{m}$ ) was calculated by dividing the sum of the perimeters of all nuclei in a field by the number of nuclei detected in that field. Highly convoluted apoptotic nuclei had the largest nuclear perimeter values. (3) The average nuclear brightness was calculated by dividing the integrated intensity of the entire field of nuclei by the number of nuclei in that field. An increase in nuclear brightness was correlated with increased DNA content. (4) The average cellular brightness was calculated by dividing the integrated intensity of an entire field of cells stained with MitoTracker dye by the number of nuclei in that field. Because the amount of MitoTracker dye that accumulates within the mitochondria is proportional to the mitochondrial potential, an increase in the average cell brightness is consistent with an increase in mitochondrial potential. (5) The average cellular brightness was also calculated by dividing the integrated intensity of an entire field of cells stained with Bodipy FL phalloidin dye by the number of nuclei in that field. Because the phallotoxins bind with high affinity to the polymerized form of actin, the amount of Bodipy FL phalloidin dye that accumulates within the cell is proportional to actin polymerization state. An increase in the average cell brightness is consistent with an increase in actin polymerization.

**Results.** Figure 24 (top panels) shows the changes paclitaxel induced in the nuclear morphology of L-929 cells. Increasing amounts of paclitaxel caused nuclei to enlarge and fragment 293, a hallmark of apoptosis. Quantitative analysis of these and other images obtained by the cell screening system is presented in the same figure.

5 Each parameter measured showed that the L-929 cells 296 were less sensitive to low concentrations of paclitaxel than were SNB-19 cells 297. At higher concentrations though, the L-929 cells showed a response for each parameter measured. The multiparameter approach of this assay is useful in dissecting the mechanisms of drug action. For example, the area, brightness, and fragmentation of the nucleus 298 and

10 actin polymerization values 294 reached a maximum value when SNB-19 cells were treated with 10 nM paclitaxel (Figure 24; top and bottom graphs). However, mitochondrial potential 295 was minimal at the same concentration of paclitaxel (Figure 24; middle graph). The fact that all the parameters measured approached control levels at increasing paclitaxel concentrations (>10 nM) suggests that SNB-19

15 cells have low affinity drug metabolic or clearance pathways that are compensatory at sufficiently high levels of the drug. Contrasting the drug sensitivity of SNB-19 cells 297, L-929 showed a different response to paclitaxel 296. These fibroblastic cells showed a maximal response in many parameters at 5  $\mu$ M paclitaxel, a 500-fold higher dose than SNB-19 cells. Furthermore, the L-929 cells did not show a sharp decrease in

20 mitochondrial potential 295 at any of the paclitaxel concentrations tested. This result is consistent with the presence of unique apoptosis pathways between a normal and cancer cell line. Therefore, these results indicate that a relatively simple fluorescence labeling protocol can be coupled with the cell screening system of the present invention to produce a high-content screen of key events involved in programmed cell death.



*Example 10. Protease induced translocation of a signaling enzyme containing a disease-associated sequence from cytoplasm to nucleus.*

5        **Plasmid construct.** A eukaryotic expression plasmid containing a coding sequence for a green fluorescent protein – caspase (Cohen (1997), *Biochemical J.* 326:1-16; Liang et al. (1997), *J. of Molec. Biol.* 274:291-302) chimera is prepared using GFP mutants. The construct is used to transfect eukaryotic cells.

**Cell preparation and transfection.** Cells are trypsinized and plated 24 h prior  
10 to transfection and incubated at 37°C and 5% CO<sub>2</sub>. Transfections are performed by methods including, but not limited to calcium phosphate coprecipitation or lipofection. Cells are incubated with the calcium phosphate-DNA precipitate for 4-5 hours at 37°C and 5% CO<sub>2</sub>, washed 3-4 times with DMEM to remove the precipitate, followed by the addition of C-DMEM. Lipofectamine transfections are performed in serum-free  
15 DMEM without antibiotics according to the manufacturer's instructions. Following a 2-3 hour incubation with the DNA-liposome complexes, the medium is removed and replaced with C-DMEM.

**Apoptotic induction of Caspase-GFP translocation.** To obtain Caspase-GFP translocation kinetic data, nuclei of transfected cells are first labeled with 5 µg/ml  
20 Hoechst 33342 (Molecular Probes) in C-DMEM for 20 minutes at 37°C and 5% CO<sub>2</sub>. Cells are washed once in Hank's Balanced Salt Solution (HBSS) followed by the addition of compounds that induce apoptosis. These compounds include, but are not limited to paclitaxel, staurosporine, ceramide, and tumor necrosis factor. To obtain fixed time point titration data, transfected cells are first washed with DMEM and then  
25 incubated at 37°C and 5% CO<sub>2</sub> for 1 h in the presence of 0 – 1000 nM compound in

DMEM. Cells are analyzed live or they are rinsed with HBSS, fixed for 15 min with 3.7% formaldehyde in HBSS, stained with Hoechst 33342, and washed before analysis.

*Image acquisition and analysis.* Kinetic data are collected by acquiring fluorescence image pairs (Caspase-GFP and Hoechst 33342-labeled nuclei) from fields of living cells at 1 min intervals for 30 min after the addition of compound. Likewise, image pairs are obtained from each well of the fixed time point screening plates 1 h after the addition of compound. In both cases, the image pairs obtained at each time point are used to define nuclear and cytoplasmic regions in each cell. Translocation of Caspase-GFP is calculated by dividing the integrated fluorescence intensity of Caspase-GFP in the nucleus by the integrated fluorescence intensity of the chimera in the cytoplasm or as a nuclear-cytoplasmic difference of GFP fluorescence. In the fixed time point screen this translocation ratio is calculated from data obtained from at least 200 cells at each concentration of compound tested. Drug-induced translocation of Caspase-GFP from the cytoplasm to the nucleus is therefore correlated with an increase in the translocation ratio. Molecular interaction libraries including, but not limited to those comprising putative activators or inhibitors of apoptosis-activated enzymes are used to screen the indicator cell lines and identify a specific ligand for the DAS, and a pathway activated by compound activity.

20 *Example 11. Identification of novel steroid receptors from DAS*

Two sources of material and/or information are required to make use of this embodiment, which allows assessment of the function of an uncharacterized gene. First, disease associated sequence bank(s) containing cDNA sequences suitable for transfection into mammalian cells can be used. Because every RADE or differential

expression experiment generates up to several hundred sequences, it is possible to generate an ample supply of DAS. Second, information from primary sequence database searches can be used to place DAS into broad categories, including, but not limited to, those that contain signal sequences, seven trans-membrane motifs, conserved protease active site domains, or other identifiable motifs. Based on the information acquired from these sources, algorithm types and indicator cell lines to be transfected are selected. A large number of motifs are already well characterized and encoded in the linear sequences contained within the large number genes in existing genomic databases.

10 In one embodiment, the following steps are taken:

- 1) Information from the DAS identification experiment (including database searches) is used as the basis for selecting the relevant biological processes. (for example, look at the DAS from a tumor line for cell cycle modulation, apoptosis, metastatic proteases, etc.)
- 15 2) Sorting of DNA sequences or DAS by identifiable motifs (ie. signal sequences, 7- transmembrane domains, conserved protease active site domains, etc.) This initial grouping will determine fluorescent tagging strategies, host cell lines, indicator cell lines, and banks of bioactive molecules to be screened, as described *supra*.
- 20 3) Using well established molecular biology methods, ligate DAS into an expression vector designed for this purpose. Generalized expression vectors contain promoters, enhancers, and terminators for which to deliver target sequences to the cell for transient expression. Such vectors may also contain antibody tagging sequences, direct association sequences, chromophore fusion sequences like GFP, etc. to facilitate  
25 detection when expressed by the host.
- 4) Transiently transfect cells with DAS containing vectors using standard transfection protocols including: calcium phosphate co-precipitation, liposome mediated, DEAE dextran mediated, polycationic mediated, viral mediated, or electroporation, and plate into microtiter plates or microwell arrays. Alternatively,  
30 transfection can be done directly in the microtiter plate itself.
- 5) Carry out the cell screening methods as described *supra*.

In this embodiment, DAS shown to possess a motif(s) suggestive of transcriptional activation potential (for example, DNA binding domain, amino terminal modulating domain, hinge region, or carboxy terminal ligand binding domain) are utilized to identify novel steroid receptors.

5       Defining the fluorescent tags for this experiment involves identification of the nucleus through staining, and tagging the DAS by creating a GFP chimera via insertion of DAS into an expression vector, proximally fused to the gene encoding GFP. Alternatively, a single chain antibody fragment with high affinity to some portion of the expressed DAS could be constructed using technology available in the art (Cambridge  
10   Antibody Technologies) and linked to a fluorophore (FITC) to tag the putative transcriptional activator/receptor in the cells. This alternative would provide an external tag requiring no DNA transfection and therefore would be useful if distribution data were to be gathered from the original primary cultures used to generate the DAS.

**Plasmid construct.** A eukaryotic expression plasmid containing a coding  
15   sequence for a green fluorescent protein – DAS chimera is prepared using GFP mutants. The construct is used to transfect HeLa cells. The plasmid, when transfected into the host cell, produces a GFP fused to the DAS protein product, designated GFP-DASpp.

**Cell preparation and transfection.** HeLa cells are trypsinized and plated using  
20   DMEM containing 5% charcoal/dextran-treated fetal bovine serum (FBS) (Hyclone) and 1% penicillin-streptomycin (C-DMEM) 12-24 hours prior to transfection and incubated at 37°C and 5% CO<sub>2</sub>. Transfections are performed by calcium phosphate coprecipitation or with Lipofectamine (Life Technologies). For the calcium phosphate transfections, the medium is replaced, prior to transfection, with DMEM containing 5%

charcoal/dextran-treated FBS. Cells are incubated with the calcium phosphate-DNA precipitate for 4-5 hours at 37°C and 5% CO<sub>2</sub>, and washed 3-4 times with DMEM to remove the precipitate, followed by the addition of C-DMEM. Lipofectamine transfections are performed in serum-free DMEM without antibiotics according to the manufacturer's instructions. Following a 2-3 hour incubation with the DNA-liposome complexes, the medium is removed and replaced with C-DMEM. All transfected cells in 96-well microtiter plates are incubated at 33°C and 5% CO<sub>2</sub> for 24-48 hours prior to drug treatment. Experiments are performed with the receptor expressed transiently in HeLa cells.

10        *Localization of expressed GFP-DASpp inside cells.* To obtain cellular distribution data, nuclei of transfected cells are first labeled with 5 µg/ml Hoechst 33342 (Molecular Probes) in C-DMEM for 20 minutes at 33°C and 5% CO<sub>2</sub>. Cells are washed once in Hank's Balanced Salt Solution (HBSS). The cells are analyzed live or they are rinsed with HBSS, fixed for 15 min with 3.7% formaldehyde in HBSS, stained  
15        with Hoechst 33342, and washed before analysis.

In a preferred embodiment, image acquisition and analysis are performed using the cell screening system of the present invention. The intracellular GFP-DASpp fluorescence signal is collected by acquiring fluorescence image pairs (GFP-DASpp and Hoechst 33342-labeled nuclei) from field cells. The image pairs obtained at each  
20        time point are used to define nuclear and cytoplasmic regions in each cell. Data demonstrating dispersed signal in the cytoplasm would be consistent with known steroid receptors that are DNA transcriptional activators.

*Screening for induction of GFP-DASpp translocation.* Using the above construct, confirmed for appropriate expression of the GFP-DASpp, as an indicator cell

line, a screen of various ligands is performed using a series of steroid type ligands including, but not limited to: estrogen, progesterone, retinoids, growth factors, androgens, and many other steroid and steroid based molecules. Image acquisition and analysis are performed using the cell screening system of the invention. The intracellular GFP-DASpp fluorescence signal is collected by acquiring fluorescence image pairs (GFP-DASpp and Hoechst 33342-labeled nuclei) from fields cells. The image pairs obtained at each time point are used to define nuclear and cytoplasmic regions in each cell. Translocation of GFP-DASpp is calculated by dividing the integrated fluorescence intensity of GFP-DASpp in the nucleus by the integrated fluorescence intensity of the chimera in the cytoplasm or as a nuclear-cytoplasmic difference of GFP fluorescence. A translocation from the cytoplasm into the nucleus indicates a ligand binding activation of the DASpp thus identifying the potential receptor class and action. Combining this data with other data obtained in a similar fashion using known inhibitors and modifiers of steroid receptors, would either validate the DASpp as a target, or more data would be generated from various sources.

*Example 12. Additional Screens*

*20 Translocation between the plasma membrane and the cytoplasm:*

**Profilactin complex dissociation and binding of profilin to the plasma membrane.** In one embodiment, a fluorescent protein biosensor of profilin membrane binding is prepared by labeling purified profilin (Federov et al.(1994), *J. Molec. Biol.* 241:480-482; Lanbrechts et al. (1995), *Eur. J. Biochem.* 230:281-286) with a probe possessing a fluorescence lifetime in the range of 2-300 ns. The labeled profilin is introduced into living indicator cells using bulk loading methodology and the indicator

cells are treated with test compounds. Fluorescence anisotropy imaging microscopy (Gough and Taylor (1993), *J. Cell Biol.* 121:1095-1107) is used to measure test-compound dependent movement of the fluorescent derivative of profilin between the cytoplasm and membrane for a period of time after treatment ranging from 0.1 s to 10 h.

**Rho-RhoGDI complex translocation to the membrane.** In another embodiment, indicator cells are treated with test compounds and then fixed, washed, and permeabilized. The indicator cell plasma membrane, cytoplasm, and nucleus are all labeled with distinctly colored markers followed by immunolocalization of Rho protein (Self et al. (1995), *Methods in Enzymology* 256:3-10; Tanaka et al. (1995), *Methods in Enzymology* 256:41-49) with antibodies labeled with a fourth color. Each of the four labels is imaged separately using the cell screening system, and the images used to calculate the amount of inhibition or activation of translocation effected by the test compound. To do this calculation, the images of the probes used to mark the plasma membrane and cytoplasm are used to mask the image of the immunological probe marking the location of intracellular Rho protein. The integrated brightness per unit area under each mask is used to form a translocation quotient by dividing the plasma membrane integrated brightness/area by the cytoplasmic integrated brightness/area. By comparing the translocation quotient values from control and experimental wells, the percent translocation is calculated for each potential lead compound.

*$\beta$ -Arrestin translocation to the plasma membrane upon G-protein receptor activation.*

In another embodiment of a cytoplasm to membrane translocation high-content screen, the translocation of  $\beta$ -arrestin protein from the cytoplasm to the plasma membrane is measured in response to cell treatment. To measure the translocation, living indicator cells containing luminescent domain markers are treated with test compounds and the movement of the  $\beta$ -arrestin marker is measured in time and space using the cell screening system of the present invention. In a preferred embodiment, the indicator cells contain luminescent markers consisting of a green fluorescent protein  $\beta$ -arrestin (GFP- $\beta$ -arrestin) protein chimera (Barak et al. (1997), *J. Biol. Chem.* 272:27497-27500; Daaka et al. (1998), *J. Biol. Chem.* 273:685-688) that is expressed by the indicator cells through the use of transient or stable cell transfection and other reporters used to mark cytoplasmic and membrane domains. When the indicator cells are in the resting state, the domain marker molecules partition predominately in the plasma membrane or in the cytoplasm. In the high-content screen, these markers are used to delineate the cell cytoplasm and plasma membrane in distinct channels of fluorescence. When the indicator cells are treated with a test compound, the dynamic redistribution of the GFP- $\beta$ -arrestin is recorded as a series of images over a time scale ranging from 0.1 s to 10 h. In a preferred embodiment, the time scale is 1 h. Each image is analyzed by a method that quantifies the movement of the GFP- $\beta$ -arrestin protein chimera between the plasma membrane and the cytoplasm. To do this calculation, the images of the probes used to mark the plasma membrane and cytoplasm are used to mask the image of the GFP- $\beta$ -arrestin probe marking the location of intracellular GFP- $\beta$ -arrestin protein. The integrated brightness per unit area under each mask is used to form a translocation quotient by dividing the plasma membrane



integrated brightness/area by the cytoplasmic integrated brightness/area. By comparing the translocation quotient values from control and experimental wells, the percent translocation is calculated for each potential lead compound. The output of the high-content screen relates quantitative data describing the magnitude of the translocation within a large number of individual cells that have been treated with test compounds of interest.

*Translocation between the endoplasmic reticulum and the Golgi:*

In one embodiment of an endoplasmic reticulum to Golgi translocation high-content screen, the translocation of a VSVG protein from the ts045 mutant strain of vesicular stomatitis virus (Ellenberg et al. (1997), *J. Cell Biol.* 138:1193-1206; Presley et al. (1997) *Nature* 389:81-85) from the endoplasmic reticulum to the Golgi domain is measured in response to cell treatment. To measure the translocation, indicator cells containing luminescent reporters are treated with test compounds and the movement of the reporters is measured in space and time using the cell screening system of the present invention. The indicator cells contain luminescent reporters consisting of a GFP-VSVG protein chimera that is expressed by the indicator cell through the use of transient or stable cell transfection and other domain markers used to measure the localization of the endoplasmic reticulum and Golgi domains. When the indicator cells are in their resting state at 40°C, the GFP-VSVG protein chimera molecules are partitioned predominately in the endoplasmic reticulum. In this high-content screen, domain markers of distinct colors used to delineate the endoplasmic reticulum and the Golgi domains in distinct channels of fluorescence. When the indicator cells are treated with a test compound and the temperature is simultaneously lowered to 32°C, the dynamic redistribution of the GFP-VSVG protein chimera is recorded as a series of

images over a time scale ranging from 0.1 s to 10 h. Each image is analyzed by a method that quantifies the movement of the GFP-VSVG protein chimera between the endoplasmic reticulum and the Golgi domains. To do this calculation, the images of the probes used to mark the endoplasmic reticulum and the Golgi domains are used to mask the image of the GFP-VSVG probe marking the location of intracellular GFP-VSVG protein. The integrated brightness per unit area under each mask is used to form a translocation quotient by dividing the endoplasmic reticulum integrated brightness/area by the Golgi integrated brightness/area. By comparing the translocation quotient values from control and experimental wells, the percent translocation is calculated for each potential lead compound. The output of the high-content screen relates quantitative data describing the magnitude of the translocation within a large number of individual cells that have been treated with test compounds of interest at final concentrations ranging from  $10^{-12}$  M to  $10^{-3}$  M for a period ranging from 1 min to 10 h.

15

*Induction and inhibition of organellar function:*

**Intracellular microtubule stability.** In one embodiment of an organellar function high-content screen, the assembly state of intracellular microtubules is measured in response to cell treatment. To measure microtubule assembly state, indicator cells containing luminescent reporters are treated with test compounds and the distribution of the reporters is measured in space and time using the cell screening system of the present invention.

In a preferred embodiment, the reporter of intracellular microtubule assembly is MAP 4 (Bulinski et al. (1997), *J. Cell Science* 110:3055-3064), a ubiquitous

microtubule associated protein that is known to interact with microtubules in interphase and mitotic cells. The indicator cells contain luminescent reporters consisting of a GFP-MAP 4 chimera that is expressed by the indicator cells through the use of transient or stable cell transfection and other reporters are used to measure the localization of the cytoplasmic and membrane components. A GFP-MAP 4 construct is prepared as follows: PCR amplification of native or mutant GFP molecules using primers to introduce restriction enzyme sites is performed. The PCR product is ligated into the MAP 4 cDNA within a eukaryotic expression vector. Indicator cells are then transfected with the expression vector to produce either transiently or stably transfected indicator cells.

Indicator cells are treated with test compounds at final concentrations ranging from  $10^{-12}$  M to  $10^{-3}$  M for a period ranging from 1 min to 10 h. Growth medium containing labeling reagent to mark the nucleus and the cytoplasm are added. After incubation, the cells are washed with Hank's balanced salt solution (HBSS), fixed with 3.7% formaldehyde for 10 min at room temperature, and washed and stored in HBSS.

Image data are obtained from both fixed and living indicator cells. To extract morphometric data from each of the images obtained the following method of analysis is used:

1. Threshold each nucleus and cytoplasmic image to produce a mask that has value = 0 for each pixel outside a nucleus or cell boundary.
2. Overlay the mask on the original image, detect each object in the field (*i.e.*, nucleus or cell), and calculate its size, shape, and integrated intensity.
3. Overlay the whole cell mask obtained above on the corresponding GFP-MAP 4 image and use an automated measurement of edge strength routine (Kolega et al.

(1993). *BioImaging* 1:136-150) to calculate the total edge strength within each cell.

To normalize for cell size, the total edge strength is divided by the cell area to give a "fibrousness" value. Large fibrousness values are associated with strong edge strength values and are therefore maximal in cells containing distinct microtubule structures. Likewise, small fibrousness values are associated with weak edge strength and are minimal in cells with depolymerized microtubules. The physiological range of fibrousness values is set by treating cells with either the microtubule stabilizing drug paclitaxel (10  $\mu$ M) or the microtubule depolymerizing drug nocodazole (10  $\mu$ g/ml).

#### *High-content screens involving the functional localization of macromolecules*

Within this class of high-content screen, the functional localization of macromolecules in response to external stimuli is measured within living cells.

**Glycolytic enzyme activity regulation.** In a preferred embodiment of a cellular enzyme activity high-content screen, the activity of key glycolytic regulatory enzymes are measured in treated cells. To measure enzyme activity, indicator cells containing luminescent labeling reagents are treated with test compounds and the activity of the reporters is measured in space and time using cell screening system of the present invention.

In one embodiment, the reporter of intracellular enzyme activity is fructose-6-phosphate, 2-kinase/fructose-2,6-bisphosphatase (PFK-2), a regulatory enzyme whose phosphorylation state indicates intracellular carbohydrate anabolism or catabolism (Deprez et al. (1997) *J. Biol. Chem.* 272:17269-17275; Kealer et al. (1996) *FEBS Letters* 395:225-227; Lee et al. (1996), *Biochemistry* 35:6010-6019). The indicator

cells contain luminescent reporters consisting of a fluorescent protein biosensor of PFK-2 phosphorylation. The fluorescent protein biosensor is constructed by introducing an environmentally sensitive fluorescent dye near to the known phosphorylation site of the enzyme (Deprez et al. (1997), *supra*; Giuliano et al. (1995), *supra*). The dye can be of the ketocyanine class (Kessler and Wolfbeis (1991), *Spectrochimica Acta* 47A:187-192 ) or any class that contains a protein reactive moiety and a fluorochrome whose excitation or emission spectrum is sensitive to solution polarity. The fluorescent protein biosensor is introduced into the indicator cells using bulk loading methodology.

Living indicator cells are treated with test compounds, at final concentrations ranging from  $10^{-12}$  M to  $10^{-3}$  M for times ranging from 0.1 s to 10 h. In a preferred embodiment, ratio image data are obtained from living treated indicator cells by collecting a spectral pair of fluorescence images at each time point. To extract morphometric data from each time point, a ratio is made between each pair of images by numerically dividing the two spectral images at each time point, pixel by pixel. Each pixel value is then used to calculate the fractional phosphorylation of PFK-2. At small fractional values of phosphorylation, PFK-2 stimulates carbohydrate catabolism. At high fractional values of phosphorylation, PFK-2 stimulates carbohydrate anabolism.

**Protein kinase A activity and localization of subunits.** In another embodiment of a high-content screen, both the domain localization and activity of protein kinase A (PKA) within indicator cells are measured in response to treatment with test compounds.

The indicator cells contain luminescent reporters including a fluorescent protein biosensor of PKA activation. The fluorescent protein biosensor is constructed by introducing an environmentally sensitive fluorescent dye into the catalytic subunit of PKA near the site known to interact with the regulatory subunit of PKA (Harootunian et al. (1993), *Mol. Biol. of the Cell* 4:993-1002; Johnson et al. (1996), *Cell* 85:149-158; Giuliano et al. (1995), *supra*). The dye can be of the ketocyanine class (Kessler, and Wolfbeis (1991), *Spectrochimica Acta* 47A:187-192) or any class that contains a protein reactive moiety and a fluorochrome whose excitation or emission spectrum is sensitive to solution polarity. The fluorescent protein biosensor of PKA activation is introduced into the indicator cells using bulk loading methodology.

In one embodiment, living indicator cells are treated with test compounds, at final concentrations ranging from  $10^{-12}$  M to  $10^{-3}$  M for times ranging from 0.1 s to 10 h. In a preferred embodiment, ratio image data are obtained from living treated indicator cells. To extract biosensor data from each time point, a ratio is made between each pair of images, and each pixel value is then used to calculate the fractional activation of PKA (e.g., separation of the catalytic and regulatory subunits after cAMP binding). At high fractional values of activity, PFK-2 stimulates biochemical cascades within the living cell.

To measure the translocation of the catalytic subunit of PKA, indicator cells containing luminescent reporters are treated with test compounds and the movement of the reporters is measured in space and time using the cell screening system. The indicator cells contain luminescent reporters consisting of domain markers used to measure the localization of the cytoplasmic and nuclear domains. When the indicator cells are treated with a test compounds, the dynamic redistribution of a PKA

fluorescent protein biosensor is recorded intracellularly as a series of images over a time scale ranging from 0.1 s to 10 h. Each image is analyzed by a method that quantifies the movement of the PKA between the cytoplasmic and nuclear domains. To do this calculation, the images of the probes used to mark the cytoplasmic and nuclear domains are used to mask the image of the PKA fluorescent protein biosensor. The integrated brightness per unit area under each mask is used to form a translocation quotient by dividing the cytoplasmic integrated brightness/area by the nuclear integrated brightness/area. By comparing the translocation quotient values from control and experimental wells, the percent translocation is calculated for each potential lead compound. The output of the high-content screen relates quantitative data describing the magnitude of the translocation within a large number of individual cells that have been treated with test compound in the concentration range of  $10^{-12}$  M to  $10^{-3}$  M.

15 *High-content screens involving the induction or inhibition of gene expression*

*RNA-based fluorescent biosensors*

**Cytoskeletal protein transcription and message localization.** Regulation of the general classes of cell physiological responses including cell-substrate adhesion, cell-cell adhesion, signal transduction, cell-cycle events, intermediary and signaling molecule metabolism, cell locomotion, cell-cell communication, and cell death can involve the alteration of gene expression. High-content screens can also be designed to measure this class of physiological response.

In one embodiment, the reporter of intracellular gene expression is an oligonucleotide that can hybridize with the target mRNA and alter its fluorescence

signal. In a preferred embodiment, the oligonucleotide is a molecular beacon (Tyagi and Kramer (1996) *Nat. Biotechnol.* 14:303-308), a luminescence-based reagent whose fluorescence signal is dependent on intermolecular and intramolecular interactions. The fluorescent biosensor is constructed by introducing a fluorescence energy transfer pair of fluorescent dyes such that there is one at each end (5' and 3') of the reagent. The dyes can be of any class that contains a protein reactive moiety and fluorochromes whose excitation and emission spectra overlap sufficiently to provide fluorescence energy transfer between the dyes in the resting state, including, but not limited to, fluorescein and rhodamine (Molecular Probes, Inc.). In a preferred embodiment, a portion of the message coding for  $\beta$ -actin (Kislauskis et al. (1994), *J. Cell Biol.* 127:441-451; McCann et al. (1997), *Proc. Natl. Acad. Sci.* 94:5679-5684; Sutoh (1982), *Biochemistry* 21:3654-3661) is inserted into the loop region of a hairpin-shaped oligonucleotide with the ends tethered together due to intramolecular hybridization. At each end of the biosensor a fluorescence donor (fluorescein) and a fluorescence acceptor (rhodamine) are covalently bound. In the tethered state, the fluorescence energy transfer is maximal and therefore indicative of an unhybridized molecule. When hybridized with the mRNA coding for  $\beta$ -actin, the tether is broken and energy transfer is lost. The complete fluorescent biosensor is introduced into the indicator cells using bulk loading methodology.

In one embodiment, living indicator cells are treated with test compounds, at final concentrations ranging from  $10^{-12}$  M to  $10^{-3}$  M for times ranging from 0.1 s to 10 h. In a preferred embodiment, ratio image data are obtained from living treated indicator cells. To extract morphometric data from each time point, a ratio is made between each pair of images, and each pixel value is then used to calculate the



fractional hybridization of the labeled nucleotide. At small fractional values of hybridization little expression of  $\beta$ -actin is indicated. At high fractional values of hybridization, maximal expression of  $\beta$ -actin is indicated. Furthermore, the distribution of hybridized molecules within the cytoplasm of the indicator cells is also a measure of the physiological response of the indicator cells.

#### *Cell surface binding of a ligand*

**Labeled insulin binding to its cell surface receptor in living cells.** Cells whose plasma membrane domain has been labeled with a labeling reagent of a particular color are incubated with a solution containing insulin molecules (Lee et al. (1997), *Biochemistry* 36:2701-2708; Martinez-Zaguilan et al. (1996), *Am. J. Physiol.* 270:C1438-C1446) that are labeled with a luminescent probe of a different color for an appropriate time under the appropriate conditions. After incubation, unbound insulin molecules are washed away, the cells fixed and the distribution and concentration of the insulin on the plasma membrane is measured. To do this, the cell membrane image is used as a mask for the insulin image. The integrated intensity from the masked insulin image is compared to a set of images containing known amounts of labeled insulin. The amount of insulin bound to the cell is determined from the standards and used in conjunction with the total concentration of insulin incubated with the cell to calculate a dissociation constant or insulin to its cell surface receptor.

#### *Labeling of cellular compartments*

##### **Whole cell labeling**

Whole cell labeling is accomplished by labeling cellular components such that dynamics of cell shape and motility of the cell can be measured over time by analyzing fluorescence images of cells.

In one embodiment, small reactive fluorescent molecules are introduced into living cells. These membrane-permeant molecules both diffuse through and react with protein components in the plasma membrane. Dye molecules react with intracellular molecules to both increase the fluorescence signal emitted from each molecule and to entrap the fluorescent dye within living cells. These molecules include reactive chloromethyl derivatives of aminocoumarins, hydroxycoumarins, eosin diacetate, fluorescein diacetate, some Bodipy dye derivatives, and tetramethylrhodamine. The reactivity of these dyes toward macromolecules includes free primary amino groups and free sulfhydryl groups.

In another embodiment, the cell surface is labeled by allowing the cell to interact with fluorescently labeled antibodies or lectins (Sigma Chemical Company, St. Louis, MO) that react specifically with molecules on the cell surface. Cell surface protein chimeras expressed by the cell of interest that contain a green fluorescent protein, or mutant thereof, component can also be used to fluorescently label the entire cell surface. Once the entire cell is labeled, images of the entire cell or cell array can become a parameter in high content screens, involving the measurement of cell shape, motility, size, and growth and division.

#### Plasma membrane labeling

In one embodiment, labeling the whole plasma membrane employs some of the same methodology described above for labeling the entire cells. Luminescent molecules that label the entire cell surface act to delineate the plasma membrane.

In a second embodiment subdomains of the plasma membrane, the extracellular surface, the lipid bilayer, and the intracellular surface can be labeled separately and used as components of high content screens. In the first embodiment, the extracellular surface is labeled using a brief treatment with a reactive fluorescent molecule such as the succinimidyl ester or iodoacetamide derivatives of fluorescent dyes such as the fluoresceins, rhodamines, cyanines, and Bodipys.

In a third embodiment, the extracellular surface is labeled using fluorescently labeled macromolecules with a high affinity for cell surface molecules. These include fluorescently labeled lectins such as the fluorescein, rhodamine, and cyanine derivatives of lectins derived from jack bean (Con A), red kidney bean (erythroagglutinin PHA-E), or wheat germ.

In a fourth embodiment, fluorescently labeled antibodies with a high affinity for cell surface components are used to label the extracellular region of the plasma membrane. Extracellular regions of cell surface receptors and ion channels are examples of proteins that can be labeled with antibodies.

In a fifth embodiment, the lipid bilayer of the plasma membrane is labeled with fluorescent molecules. These molecules include fluorescent dyes attached to long chain hydrophobic molecules that interact strongly with the hydrophobic region in the center of the plasma membrane lipid bilayer. Examples of these dyes include the PKH series of dyes (U.S. 4,783,401, 4,762,701, and 4,859,584; available commercially from Sigma Chemical Company, St. Louis, MO), fluorescent phospholipids such as

nitrobenzoxadiazole glycerophosphoethanolamine and fluorescein-derivatized dihexadecanoylglycerophosphoethanolamine, fluorescent fatty acids such as 5-butyl-4,4-difluoro-4-bora-3a,4a-diaza-s-indacene-3-nonanoic acid and 1-pyrenedecanoic acid (Molecular Probes, Inc.), fluorescent sterols including cholesteryl 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoate and cholesteryl 1-pyrenehexanoate, and fluorescently labeled proteins that interact specifically with lipid bilayer components such as the fluorescein derivative of annexin V (Caltag Antibody Co, Burlingame, CA).

In another embodiment, the intracellular component of the plasma membrane is labeled with fluorescent molecules. Examples of these molecules are the intracellular components of the trimeric G-protein receptor, adenylyl cyclase, and ionic transport proteins. These molecules can be labeled as a result of tight binding to a fluorescently labeled specific antibody or by the incorporation of a fluorescent protein chimera that is comprised of a membrane-associated protein and the green fluorescent protein, and mutants thereof.

#### **Endosome fluorescence labeling**

In one embodiment, ligands that are transported into cells by receptor-mediated endocytosis are used to trace the dynamics of endosomal organelles. Examples of labeled ligands include Bodipy FL-labeled low density lipoprotein complexes, tetramethylrhodamine transferrin analogs, and fluorescently labeled epidermal growth factor (Molecular Probes, Inc.)

In a second embodiment, fluorescently labeled primary or secondary antibodies (Sigma Chemical Co. St. Louis, MO; Molecular Probes, Inc. Eugene, OR; Caltag

Antibody Co.) that specifically label endosomal ligands are used to mark the endosomal compartment in cells.

In a third embodiment, endosomes are fluorescently labeled in cells expressing protein chimeras formed by fusing a green fluorescent protein, or mutants thereof, with a receptor whose internalization labels endosomes. Chimeras of the EGF, transferrin, and low density lipoprotein receptors are examples of these molecules.

### **Lysosome labeling**

In one embodiment, membrane permeant lysosome-specific luminescent reagents are used to label the lysosomal compartment of living and fixed cells. These reagents include the luminescent molecules neutral red, N-(3-((2,4-dinitrophenyl)amino)propyl)-N-(3-aminopropyl)methylamine, and the LysoTracker probes which report intralysosomal pH as well as the dynamic distribution of lysosomes (Molecular Probes, Inc.)

In a second embodiment, antibodies against lysosomal antigens (Sigma Chemical Co.; Molecular Probes, Inc.; Caltag Antibody Co.) are used to label lysosomal components that are localized in specific lysosomal domains. Examples of these components are the degradative enzymes involved in cholesterol ester hydrolysis, membrane protein proteases, and nucleases as well as the ATP-driven lysosomal proton pump.

In a third embodiment, protein chimeras consisting of a lysosomal protein genetically fused to an intrinsically luminescent protein such as the green fluorescent protein, or mutants thereof, are used to label the lysosomal domain. Examples of these components are the degradative enzymes involved in cholesterol ester hydrolysis,

membrane protein proteases, and nucleases as well as the ATP-driven lysosomal proton pump.

### **Cytoplasmic fluorescence labeling**

5        In one embodiment, cell permeant fluorescent dyes (Molecular Probes, Inc.) with a reactive group are reacted with living cells. Reactive dyes including monobromobimane, 5-chloromethylfluorescein diacetate, carboxy fluorescein diacetate succinimidyl ester, and chloromethyl tetramethylrhodamine are examples of cell permeant fluorescent dyes that are used for long term labeling of the cytoplasm of cells.

10       In a second embodiment, polar tracer molecules such as Lucifer yellow and cascade blue-based fluorescent dyes (Molecular Probes, Inc.) are introduced into cells using bulk loading methods and are also used for cytoplasmic labeling.

      In a third embodiment, antibodies against cytoplasmic components (Sigma Chemical Co.; Molecular Probes, Inc.; Caltag Antibody Co.) are used to fluorescently  
15       label the cytoplasm. Examples of cytoplasmic antigens are many of the enzymes involved in intermediary metabolism. Enolase, phosphofructokinase, and acetyl-CoA dehydrogenase are examples of uniformly distributed cytoplasmic antigens.

      In a fourth embodiment, protein chimeras consisting of a cytoplasmic protein genetically fused to an intrinsically luminescent protein such as the green fluorescent  
20       protein, or mutants thereof, are used to label the cytoplasm. Fluorescent chimeras of uniformly distributed proteins are used to label the entire cytoplasmic domain. Examples of these proteins are many of the proteins involved in intermediary metabolism and include enolase, lactate dehydrogenase, and hexokinase.

In a fifth embodiment, antibodies against cytoplasmic antigens (Sigma Chemical Co.; Molecular Probes, Inc.; Caltag Antibody Co.) are used to label cytoplasmic components that are localized in specific cytoplasmic sub-domains. Examples of these components are the cytoskeletal proteins actin, tubulin, and  
5 cytokeratin. A population of these proteins within cells is assembled into discrete structures, which in this case, are fibrous. Fluorescence labeling of these proteins with antibody-based reagents therefore labels a specific sub-domain of the cytoplasm.

In a sixth embodiment, non-antibody-based fluorescently labeled molecules that interact strongly with cytoplasmic proteins are used to label specific cytoplasmic  
10 components. One example is a fluorescent analog of the enzyme DNase I (Molecular Probes, Inc.) Fluorescent analogs of this enzyme bind tightly and specifically to cytoplasmic actin, thus labeling a sub-domain of the cytoplasm. In another example, fluorescent analogs of the mushroom toxin phalloidin or the drug paclitaxel (Molecular Probes, Inc.) are used to label components of the actin- and microtubule-cytoskeletons,  
15 respectively.

In a seventh embodiment, protein chimeras consisting of a cytoplasmic protein genetically fused to an intrinsically luminescent protein such as the green fluorescent protein, or mutants thereof, are used to label specific domains of the cytoplasm. Fluorescent chimeras of highly localized proteins are used to label cytoplasmic sub-  
20 domains. Examples of these proteins are many of the proteins involved in regulating the cytoskeleton. They include the structural proteins actin, tubulin, and cytokeratin as well as the regulatory proteins microtubule associated protein 4 and  $\alpha$ -actinin.

### Nuclear labeling

In one embodiment, membrane permeant nucleic-acid-specific luminescent reagents (Molecular Probes, Inc.) are used to label the nucleus of living and fixed cells. These reagents include cyanine-based dyes (*e.g.*, TOTO<sup>®</sup>, YOYO<sup>®</sup>, and BOBO<sup>™</sup>), phenanthridines and acridines (*e.g.*, ethidium bromide, propidium iodide, and acridine orange), indoles and imidazoles (*e.g.*, Hoechst 33258, Hoechst 33342, and 4',6-diamidino-2-phenylindole), and other similar reagents (*e.g.*, 7-aminoactinomycin D, hydroxystilbamidine, and the psoralens).

In a second embodiment, antibodies against nuclear antigens (Sigma Chemical Co.; Molecular Probes, Inc.; Caltag Antibody Co.) are used to label nuclear components that are localized in specific nuclear domains. Examples of these components are the macromolecules involved in maintaining DNA structure and function. DNA, RNA, histones, DNA polymerase, RNA polymerase, lamins, and nuclear variants of cytoplasmic proteins such as actin are examples of nuclear antigens.

In a third embodiment, protein chimeras consisting of a nuclear protein genetically fused to an intrinsically luminescent protein such as the green fluorescent protein, or mutants thereof, are used to label the nuclear domain. Examples of these proteins are many of the proteins involved in maintaining DNA structure and function. Histones, DNA polymerase, RNA polymerase, lamins, and nuclear variants of cytoplasmic proteins such as actin are examples of nuclear proteins.

### Mitochondrial labeling

In one embodiment, membrane permeant mitochondrial-specific luminescent reagents (Molecular Probes, Inc.) are used to label the mitochondria of living and fixed



cells. These reagents include rhodamine 123, tetramethyl rosamine, JC-1, and the MitoTracker reactive dyes.

In a second embodiment, antibodies against mitochondrial antigens (Sigma Chemical Co.; Molecular Probes, Inc.; Caltag Antibody Co.) are used to label  
5 mitochondrial components that are localized in specific mitochondrial domains. Examples of these components are the macromolecules involved in maintaining mitochondrial DNA structure and function. DNA, RNA, histones, DNA polymerase, RNA polymerase, and mitochondrial variants of cytoplasmic macromolecules such as mitochondrial tRNA and rRNA are examples mitochondrial antigens. Other examples  
10 of mitochondrial antigens are the components of the oxidative phosphorylation system found in the mitochondria (*e.g.*, cytochrome c, cytochrome c oxidase, and succinate dehydrogenase).

In a third embodiment, protein chimeras consisting of a mitochondrial protein genetically fused to an intrinsically luminescent protein such as the green fluorescent  
15 protein, or mutants thereof, are used to label the mitochondrial domain. Examples of these components are the macromolecules involved in maintaining mitochondrial DNA structure and function. Examples include histones, DNA polymerase, RNA polymerase, and the components of the oxidative phosphorylation system found in the mitochondria (*e.g.*, cytochrome c, cytochrome c oxidase, and succinate  
20 dehydrogenase).

#### **Endoplasmic reticulum labeling**

In one embodiment, membrane permeant endoplasmic reticulum-specific luminescent reagents (Molecular Probes, Inc.) are used to label the endoplasmic reticulum of living and fixed cells. These reagents include short chain carbocyanine

dyes (*e.g.*, DiOC<sub>6</sub> and DiOC<sub>3</sub>), long chain carbocyanine dyes (*e.g.*, DiIC<sub>16</sub> and DiIC<sub>18</sub>), and luminescently labeled lectins such as concanavalin A.

In a second embodiment, antibodies against endoplasmic reticulum antigens (Sigma Chemical Co.; Molecular Probes, Inc.; Caltag Antibody Co.) are used to label  
5 endoplasmic reticulum components that are localized in specific endoplasmic reticulum domains. Examples of these components are the macromolecules involved in the fatty acid elongation systems, glucose-6-phosphatase, and HMG CoA-reductase.

In a third embodiment, protein chimeras consisting of a endoplasmic reticulum protein genetically fused to an intrinsically luminescent protein such as the green  
10 fluorescent protein, or mutants thereof, are used to label the endoplasmic reticulum domain. Examples of these components are the macromolecules involved in the fatty acid elongation systems, glucose-6-phosphatase, and HMG CoA-reductase.

#### **Golgi labeling**

In one embodiment, membrane permeant Golgi-specific luminescent reagents  
15 (Molecular Probes, Inc.) are used to label the Golgi of living and fixed cells. These reagents include luminescently labeled macromolecules such as wheat germ agglutinin and Brefeldin A as well as luminescently labeled ceramide.

In a second embodiment, antibodies against Golgi antigens (Sigma Chemical Co.; Molecular Probes, Inc.; Caltag Antibody Co.) are used to label Golgi components  
20 that are localized in specific Golgi domains. Examples of these components are N-acetylglucosamine phosphotransferase, Golgi-specific phosphodiesterase, and mannose-6-phosphate receptor protein.

In a third embodiment, protein chimeras consisting of a Golgi protein genetically fused to an intrinsically luminescent protein such as the green fluorescent

protein, or mutants thereof, are used to label the Golgi domain. Examples of these components are N-acetylglucosamine phosphotransferase, Golgi-specific phosphodiesterase, and mannose-6-phosphate receptor protein.

While many of the examples presented involve the measurement of single  
5 cellular processes, this is again is intended for purposes of illustration only. Multiple parameter high-content screens can be produced by combining several single parameter screens into a multiparameter high-content screen or by adding cellular parameters to any existing high-content screen. Furthermore, while each example is described as being based on either live or fixed cells, each high-content screen can be designed to be  
10 used with both live and fixed cells.

Those skilled in the art will recognize a wide variety of distinct screens that can be developed based on the disclosure provided herein. There is a large and growing list of known biochemical and molecular processes in cells that involve translocations or reorganizations of specific components within cells. The signaling pathway from the  
15 cell surface to target sites within the cell involves the translocation of plasma membrane-associated proteins to the cytoplasm. For example, it is known that one of the src family of protein tyrosine kinases, pp60c-src (Walker et al (1993), *J. Biol. Chem.* 268:19552-19558) translocates from the plasma membrane to the cytoplasm upon stimulation of fibroblasts with platelet-derived growth factor (PDGF).  
20 Additionally, the targets for screening can themselves be converted into fluorescence-based reagents that report molecular changes including ligand-binding and post-translocational modifications.

We claim:

1. An automated method for identifying compounds that induce internalization of cell surface receptor proteins comprising
  - 5 -providing an array of locations containing multiple cells to be treated with a test compound, wherein the cells possess a cell surface receptor protein of interest, and wherein the cell surface receptor protein is either expressed as a luminescently labeled protein, or is luminescently labeled by contacting the cell with a luminescently labeled molecule that binds to the cell surface receptor of interest, wherein the contacting can  
10 be carried out either before or after treatment with the test compound;
    - treating the cells with the test compound;
    - scanning multiple cells in each of the locations containing multiple cells to obtain luminescent signals from the luminescently-labeled cell surface receptor protein;
    - converting the luminescent signals into digital data; and  
15 -utilizing the digital data to automatically determine whether the test compound has induced internalization of the luminescently labeled cell surface receptor protein.
2. The method of claim 1 further comprising determining a number of cells that have internalized the luminescently labeled cell surface receptor protein.  
20
3. The method of claim 2 further comprising determining a total cell number.
4. The method of claim 3 wherein the determining of the total cell number comprises the steps of:

- a. acquiring an image of the cell nuclei;
  - b. segmenting the image of the cell nuclei; and
  - c. calculating the total area of all the nuclei in the image of the cell nuclei.
- 5 5. The method of claim 2 wherein the determining a number of cells that have internalized the luminescently labeled cell surface receptor protein comprises the steps of:
- a. acquiring an object image of the luminescently labeled cell surface receptor protein in or on the cells;
  - 10 b. segmenting the object image; and
  - c. determining whether objects in the segmented object image represent valid internalized luminescently labeled cell surface receptor proteins.
6. The method of claim 5 further comprising at least one of the following:
- 15 a. removing artifacts from the object image; or
  - b. correcting for background luminescence.
7. The method of claim 5 further comprising measuring at least one of the following:
- 20 (a) a number of objects that were determined to represent valid internalized cell surface receptors;
  - (b) an aggregate area of the objects that were determined to represent valid internalized cell surface receptors;

(c) an aggregate intensity of the objects that were determined to represent valid internalized cell surface receptors; or

(d) a normalized aggregate intensity of the objects that were determined to represent valid internalized cell surface receptors.

5

8. The method of claim 1 wherein sub-regions of the array of locations containing multiple cells are sampled multiple times at intervals to provide kinetic measurement of cell surface receptor protein internalization into the cell.

10 9. A method for identifying compounds that inhibit internalization of cell surface receptor proteins comprising

-providing an array of locations containing multiple cells to be treated with a test compound, wherein the cells possess a cell surface receptor protein of interest, and wherein the cell surface receptor protein is either expressed as a luminescently labeled protein, or is luminescently labeled by contacting the cell with a luminescently labeled molecule that binds to the cell surface receptor of interest, wherein the contacting can be carried out either before or after treatment with the test compound;

-treating the cells with a test compound;

-treating the cells with a ligand that causes the cell surface receptor protein to be internalized in the absence of the test compound;

20

-scanning multiple cells in each of the locations containing cells to obtain luminescent signals from the luminescently labeled receptor protein;

-converting the luminescent signals into digital data; and

-utilizing the digital data to determine whether the test compound has inhibited ligand-induced internalization of the luminescently labeled cell surface receptor protein into the cell.

- 5 10. The method of claim 9 further comprising determining a number of cells that have internalized the luminescently labeled cell surface receptor protein.
11. The method of claim 10 further comprising determining a total cell number.
- 10 12. The method of claim 11 wherein the determining of the total cell number comprises the steps of:
- a. acquiring an image of the cell nuclei;
  - b. segmenting the image of the cell nuclei; and
  - c. calculating the total area of all the nuclei in the image of the cell nuclei.
- 15 13. The method of claim 10 wherein the determining a number of cells that have internalized the luminescently labeled receptor protein comprises the steps of:
- a. acquiring an object image of the luminescently labeled cell surface receptor protein in or on the cells;
  - 20 b. segmenting the object image; and
  - c. determining whether objects in the segmented object image represent valid internalized luminescently labeled cell surface receptor proteins.
14. The method of claim 13 further comprising at least one of the following:

- a. removing artifacts from the object image; or
- b. correcting for background luminescence.

15. The method of claim 13 further comprising measuring at least one of the  
5 following:

- (a) a number of objects that were determined to represent valid internalized cell surface receptors;
- (b) an aggregate area of the objects that were determined to represent valid internalized cell surface receptors;
- 10 (c) an aggregate intensity of the objects that were determined to represent valid internalized cell surface receptors; or
- (d) a normalized aggregate intensity of the objects that were determined to represent valid internalized cell surface receptors.

15 16. The method of claim 9 wherein sub-regions of the array of locations containing multiple cells are sampled multiple times at intervals to provide kinetic measurement of the inhibition of cell surface receptor protein internalization into cell.

17. A method for identifying compounds that induce internalization of cell surface  
20 receptor proteins comprising

-providing an array of locations containing multiple cells to be treated with a test compound, wherein the cells possess a cell surface receptor protein of interest, and wherein the cell surface receptor protein is either expressed as a luminescently labeled protein, or is luminescently labeled by contacting the cell with a luminescently labeled



molecule that binds to the cell surface receptor of interest, wherein the contacting can be carried out either before or after treatment with the test compound;

-treating the cells with an indicator that produces a detectable signal upon stimulation of the receptor protein

5        -treating the cells with a test compound;

-scanning the cells in a high throughput mode to identify those cells that exhibit the detectable signal;

-selectively scanning only a subset of the cells in a high content mode to obtain luminescent signals from the luminescently labeled receptor protein, wherein the subset  
10 consists of the cells that exhibit the detectable signal during scanning in the high throughput mode;

-converting the luminescent signals into digital data; and

-utilizing the digital data to determine whether the test compound has induced internalization of the luminescently labeled cell surface receptor into the cell.

15

18. The method of claim 17 further comprising determining a number of cells that have internalized the luminescently labeled cell surface receptor protein.

19. The method of claim 18 further comprising determining a total cell number.

20

20. The method of claim 19 wherein the determining of the total cell number comprises the steps of:

- a. acquiring an image of the cell nuclei;
- b. segmenting the image of the cell nuclei; and

- c, calculating the total area of all the nuclei in the image of the cell nuclei.

21. The method of claim 18 wherein the determining a number of cells that have internalized the luminescently labeled receptor protein comprises the steps of:

- 5           a. acquiring an object image of the luminescently labeled cell surface receptor protein in or on the cells;
- b. segmenting the object image; and
- c. determining whether objects in the segmented object image represent valid internalized luminescently labeled cell surface receptor proteins.

10

22. The method of claim 21 further comprising at least one of the following:

- a. removing artifacts from the object image; or
- b. correcting for background luminescence.

15   23. The method of claim 21 further comprising measuring at least one of the following:

- (a) a number of objects that were determined to represent valid internalized cell surface receptors;
- (b) an aggregate area of the objects that were determined to represent valid internalized cell surface receptors;
- 20           (c) an aggregate intensity of the objects that were determined to represent valid internalized cell surface receptors; or
- (d) a normalized aggregate intensity of the objects that were determined to represent valid internalized cell surface receptors.

24. The method of claim 17 wherein sub-regions of the array of locations containing multiple cells are sampled multiple times at intervals to provide kinetic measurement of cell surface receptor internalization.

5

25. A method for identifying compounds that inhibit internalization of cell surface receptor proteins comprising

-providing an array of locations containing multiple cells to be treated with a test compound, wherein the cells possess a cell surface receptor protein of interest, and  
10 wherein the cell surface receptor protein is either expressed as a luminescently labeled protein, or is luminescently labeled by contacting the cell with a luminescently labeled molecule that binds to the cell surface receptor of interest, wherein the contacting can be carried out either before or after treatment with the test compound;

-treating the cells with an indicator that produces a detectable signal upon  
15 stimulation of the receptor protein

-treating the cells with a test compound;

-treating the cells with a ligand that causes the cell surface receptor protein to be internalized into the cell in the absence of the test compound;

-scanning the cells in a high throughput mode to identify those cells that do not  
20 exhibit the indicator-induced detectable signal;

-selectively scanning only a subset of the cells in a high content mode to obtain luminescent signals from the luminescently labeled receptor protein, wherein the subset consists of the cells that did not exhibit the desired detectable signal during scanning in the high throughput mode;

-converting the luminescent signals into digital data; and  
-utilizing the digital data to determine whether the test compound has inhibited  
ligand-induced internalization of the luminescently labeled cell surface receptor protein  
into the cell.

5

26. The method of claim 25 further comprising determining a number of cells that  
have internalized the luminescently labeled cell surface receptor protein.

27. The method of claim 26 further comprising determining a total cell number.

10

28. The method of claim 27 wherein the determining of the cell number comprises  
the steps of:

- a. acquiring an image of the cell nuclei;
- b. segmenting the image of the cell nuclei; and
- 15 c, calculating the total area of all the nuclei in the image of the cell nuclei.

29. The method of claim 26 wherein the determining a number of cells that have  
internalized the luminescently labeled receptor protein comprises the steps of:

- a. acquiring an object image of the luminescently labeled cell surface  
20 receptor protein in or on the cells;
- b. segmenting the object image; and
- c. determining whether objects in the segmented object image represent  
valid internalized luminescently labeled cell surface receptor proteins.

30. The method of claim 29 further comprising at least one of the following:
- a. removing artifacts from the object image; and
  - b. correcting for background luminescence.
- 5 31. The method of claim 29 further comprising measuring at least one of the following:
- (a) a number of objects that were determined to represent valid internalized cell surface receptors;
  - (b) an aggregate area of the objects that were determined to represent valid  
10 internalized cell surface receptors;
  - (c) an aggregate intensity of the objects that were determined to represent valid internalized cell surface receptors; or
  - (d) a normalized aggregate intensity of the objects that were determined to represent valid internalized cell surface receptors.
- 15 32. The method of claim 25 wherein sub-regions of the array of locations containing multiple cells are sampled multiple times at intervals to provide kinetic measurement of cell surface receptor internalization.
- 20 33. A computer readable storage medium comprising a program containing a set of instructions for causing a cell screening system to execute the method of claim 1 wherein the cell screening system comprises an optical system with a stage adapted for holding a plate containing cells, a means for moving the stage or the optical system, a digital camera, a means for directing light emitted from the cells to the digital camera,

and a computer means for receiving and processing the digital data from the digital camera.

34. A computer readable storage medium comprising a program containing a set of  
5 instructions for causing a cell screening system to execute the method of claim 9  
wherein the cell screening system comprises an optical system with a stage adapted for  
holding a plate containing cells, a means for moving the stage or the optical system, a  
digital camera, a means for directing light emitted from the cells to the digital camera,  
and a computer means for receiving and processing the digital data from the digital  
10 camera.

35. A computer readable storage medium comprising a program containing a set of  
instructions for causing a cell screening system to execute the method of claim 17  
wherein the cell screening system comprises an optical system with a stage adapted for  
15 holding a plate containing cells, a means for moving the stage or the optical system, a  
digital camera, a means for directing light emitted from the cells to the digital camera,  
and a computer means for receiving and processing the digital data from the digital  
camera.

20 36. A computer readable storage medium comprising a program containing a set of  
instructions for causing a cell screening system to execute the method of claim 25  
wherein the cell screening system comprises an optical system with a stage adapted for  
holding a plate containing cells, a means for moving the stage or the optical system, a  
digital camera, a means for directing light emitted from the cells to the digital camera,

and a computer means for receiving and processing the digital data from the digital camera.

37. A kit for identifying compounds that induce or inhibit internalization of cell  
5 surface receptor proteins comprising:

(a) at least one antibody or fragment thereof that specifically binds to a cell  
surface receptor protein of interest; and

(b) instructions for using the antibody to identify compounds that induce or  
inhibit internalization of the cell surface receptor protein of interest into cells.

10

38. The kit of claim 37 wherein the cell surface receptor protein of interest is a G-  
protein coupled receptor.

39. The kit of claim 38 wherein the G-protein coupled receptor is parathyroid  
15 hormone receptor.

20

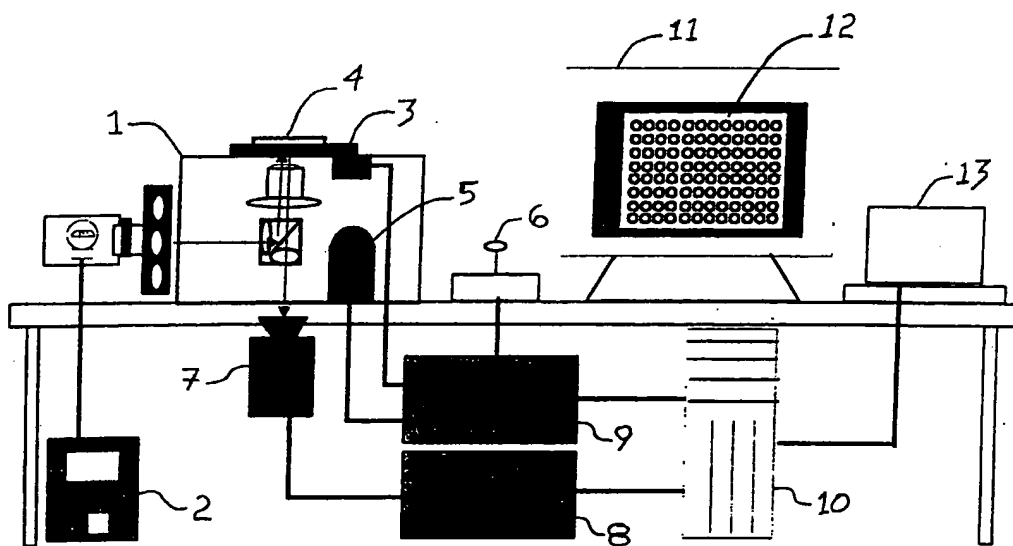


Fig 1



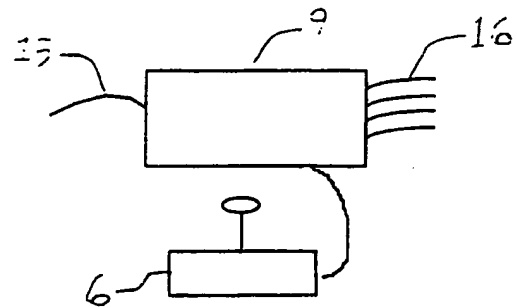
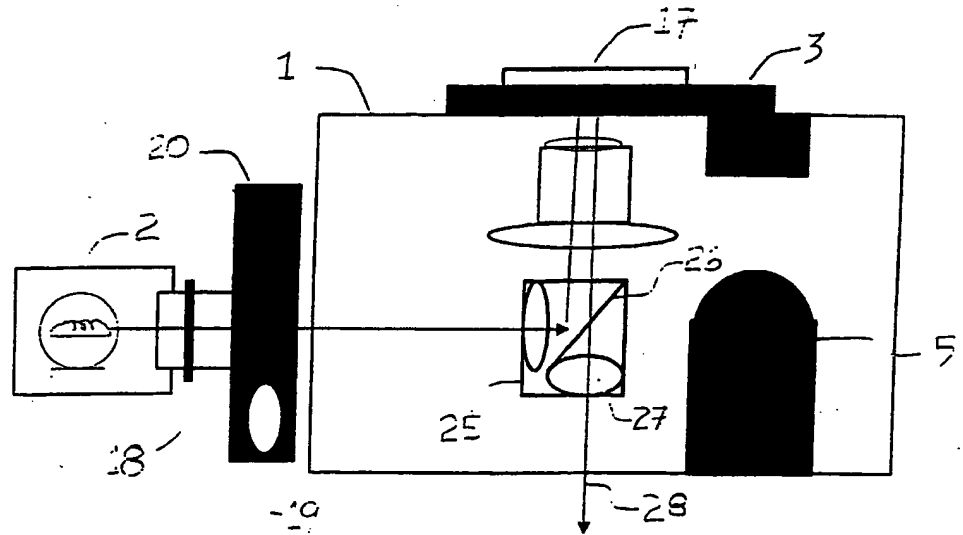


Fig. 2

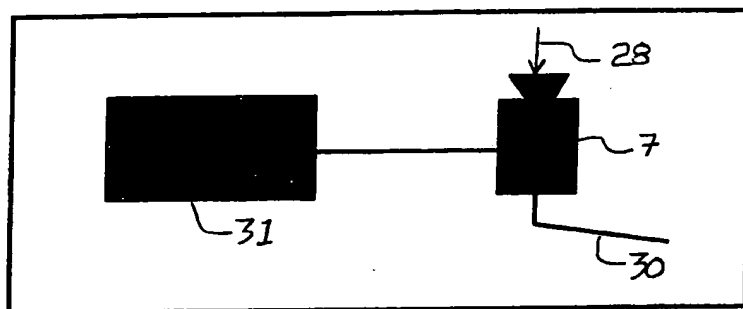


Fig 3

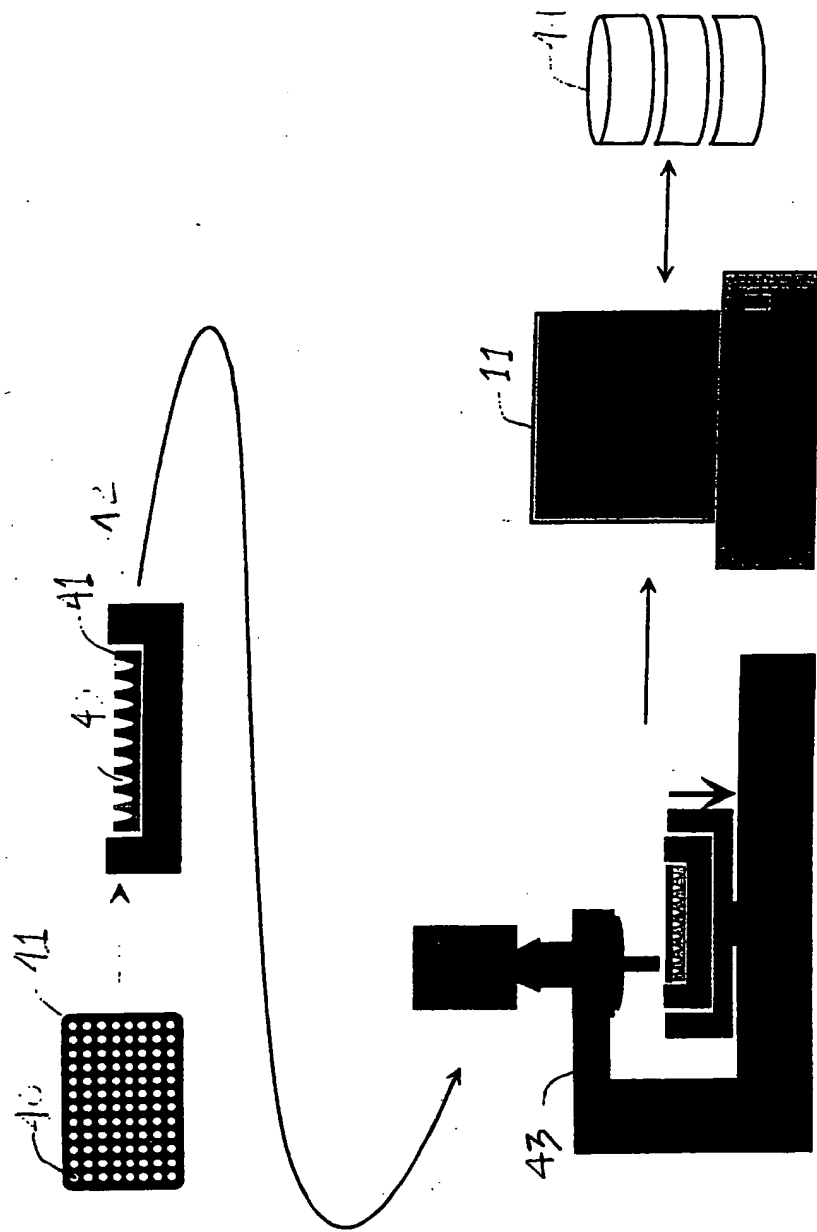


Fig. 4

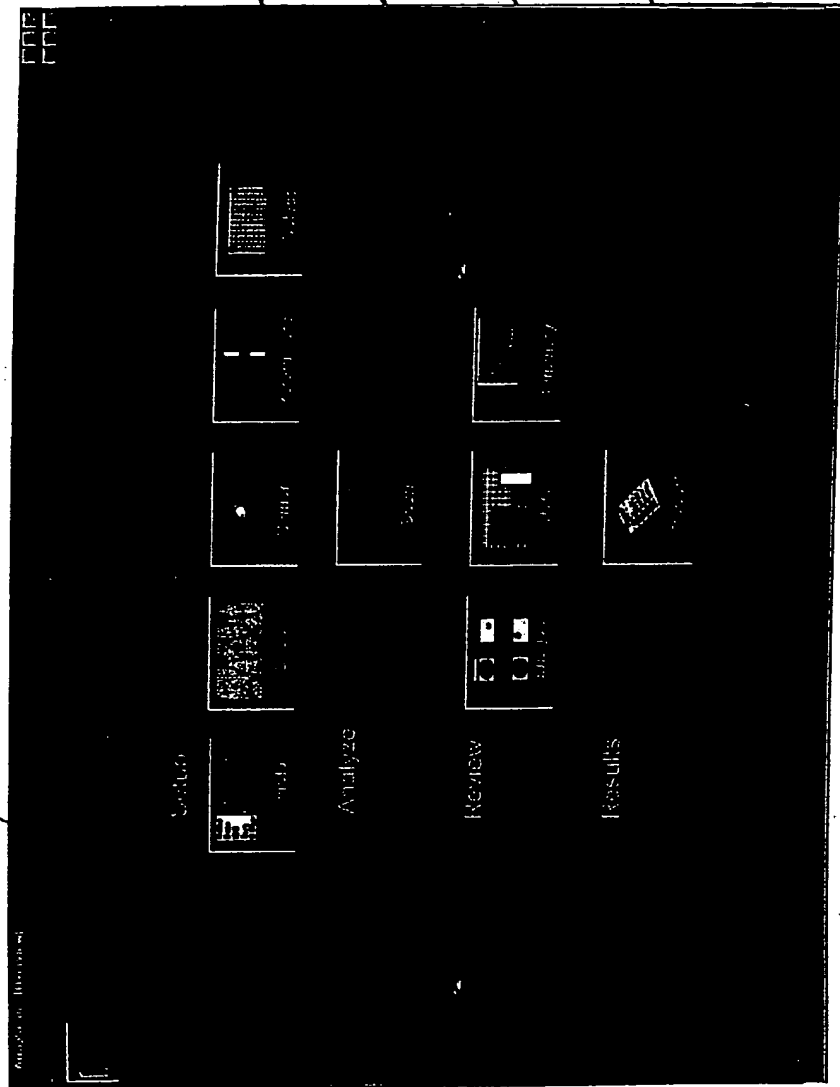


Fig 5

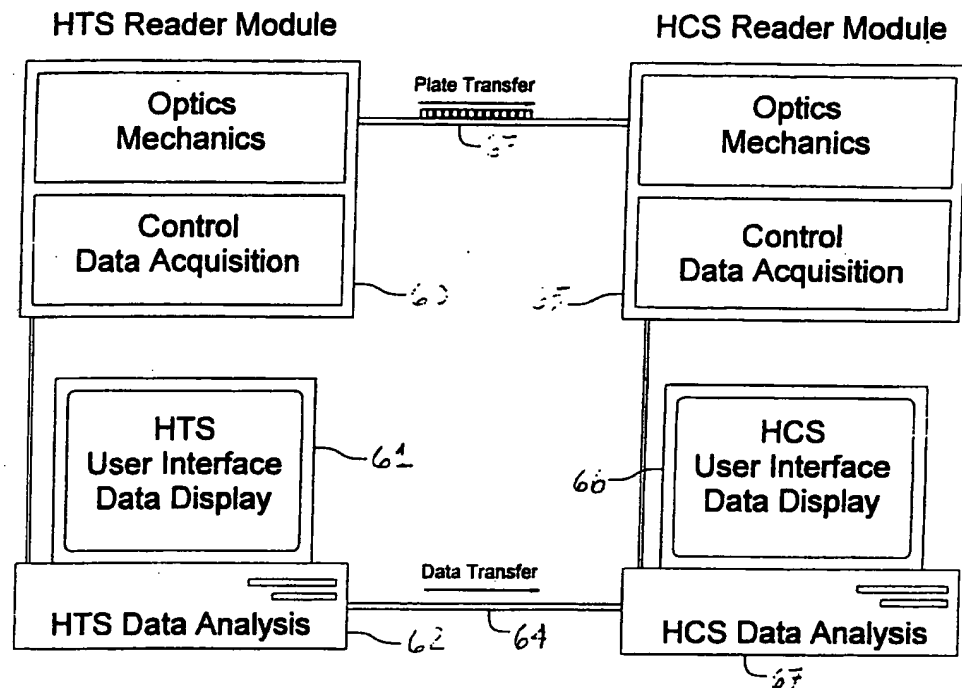


Fig. 6

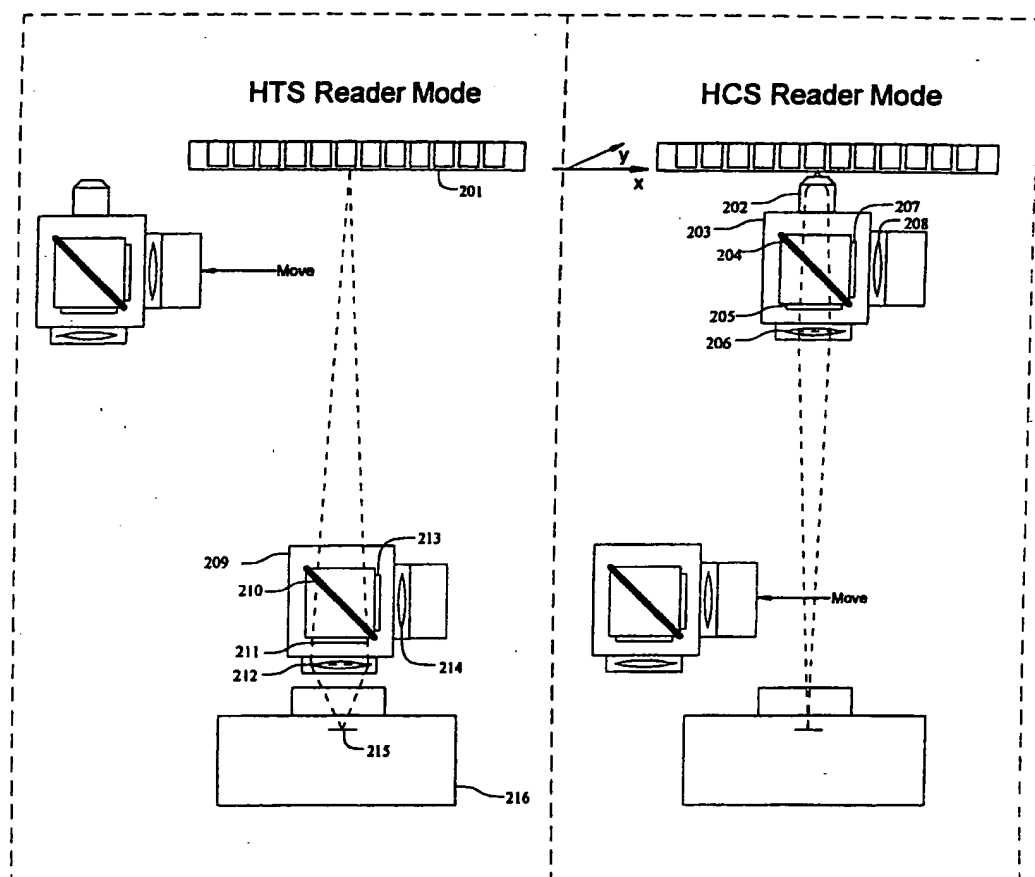


Fig. 7

# Fluid Delivery System for Cell Based Screening System

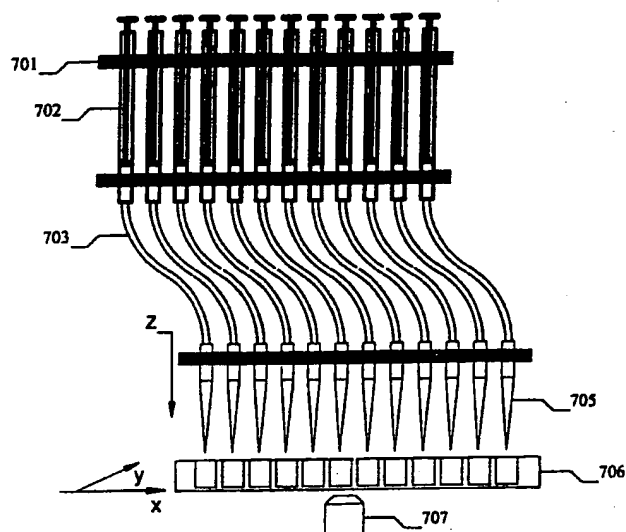


Fig. 8

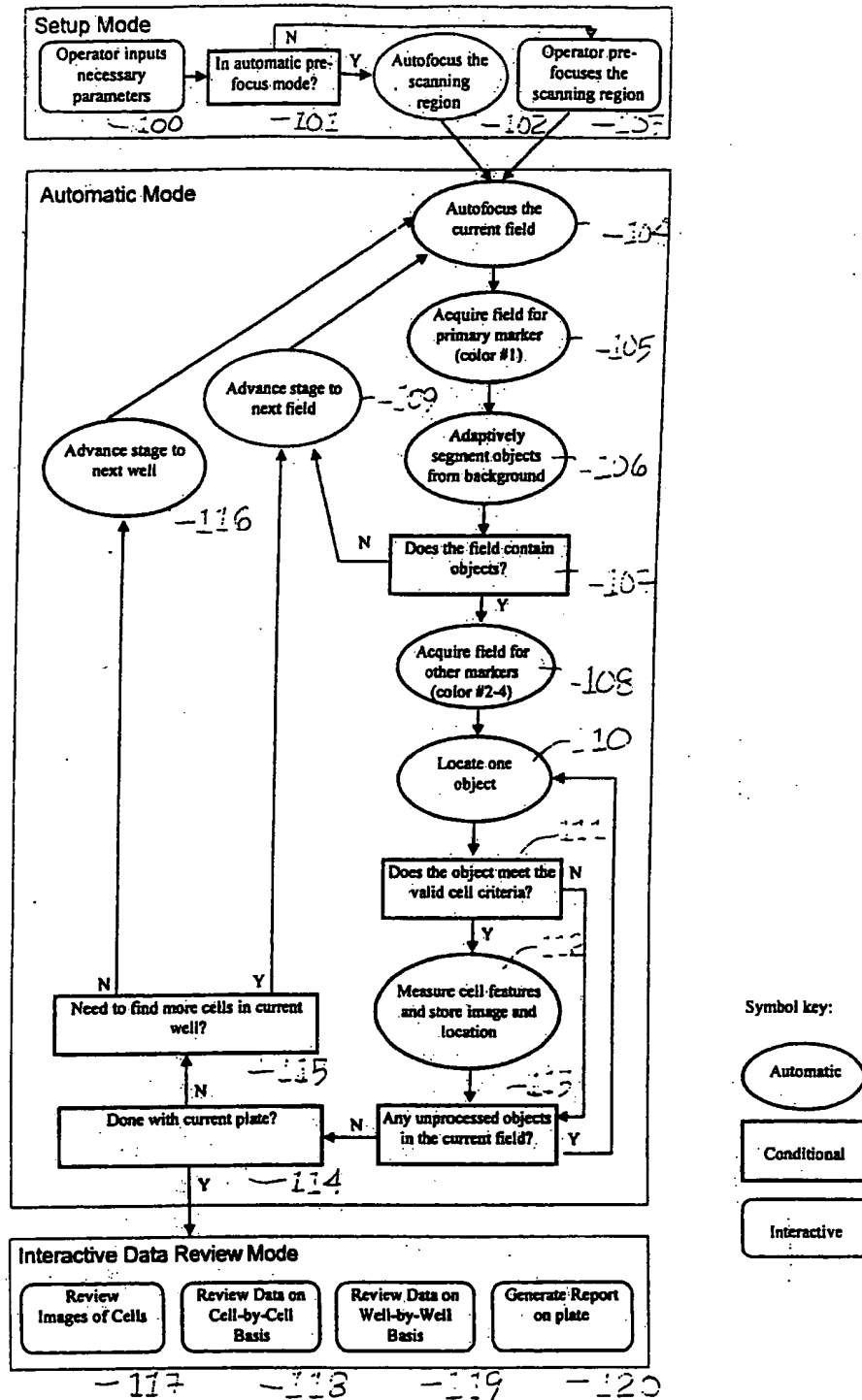


Fig 9



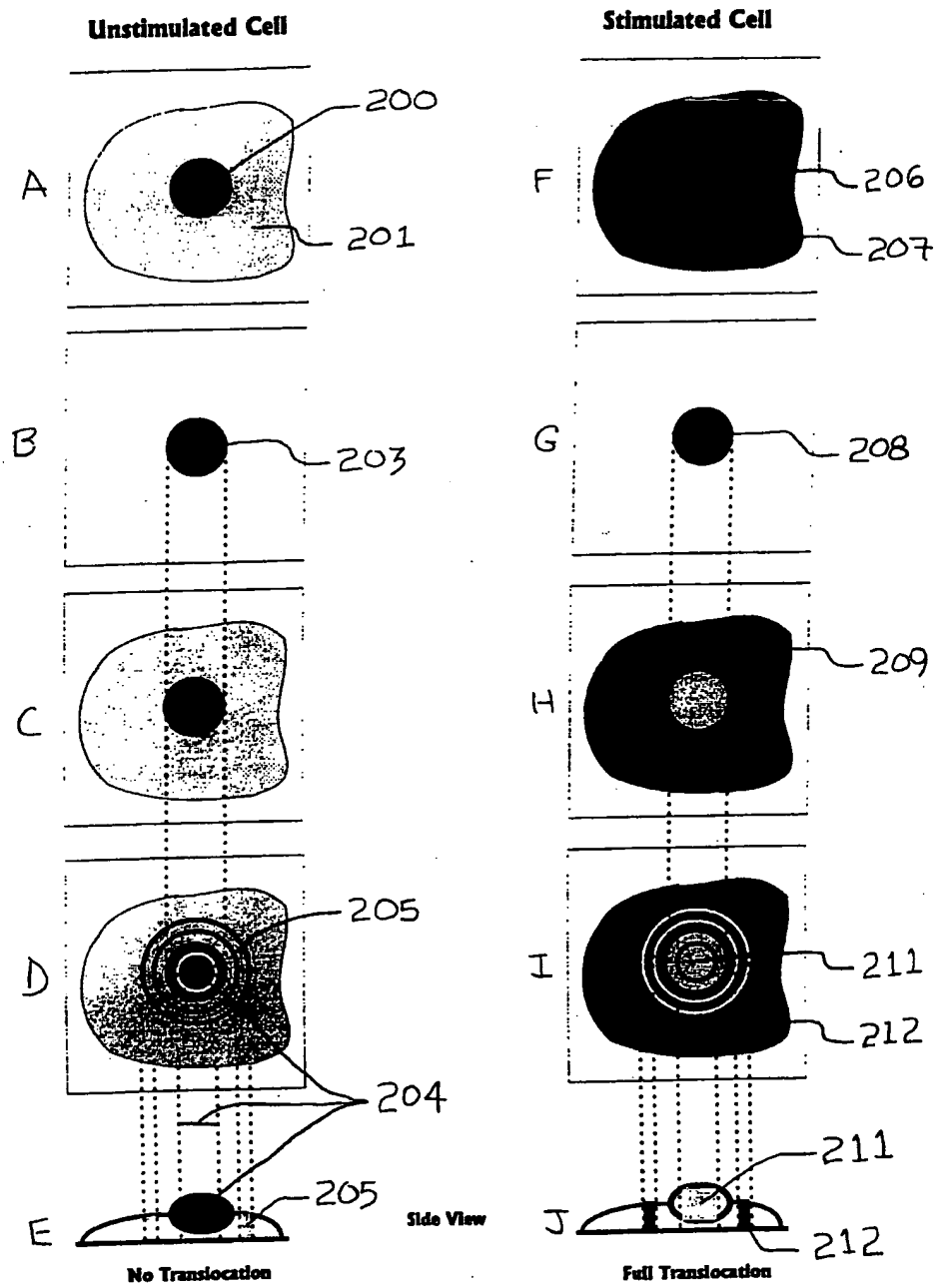


Fig 10

## Dual Mode Processing Overview

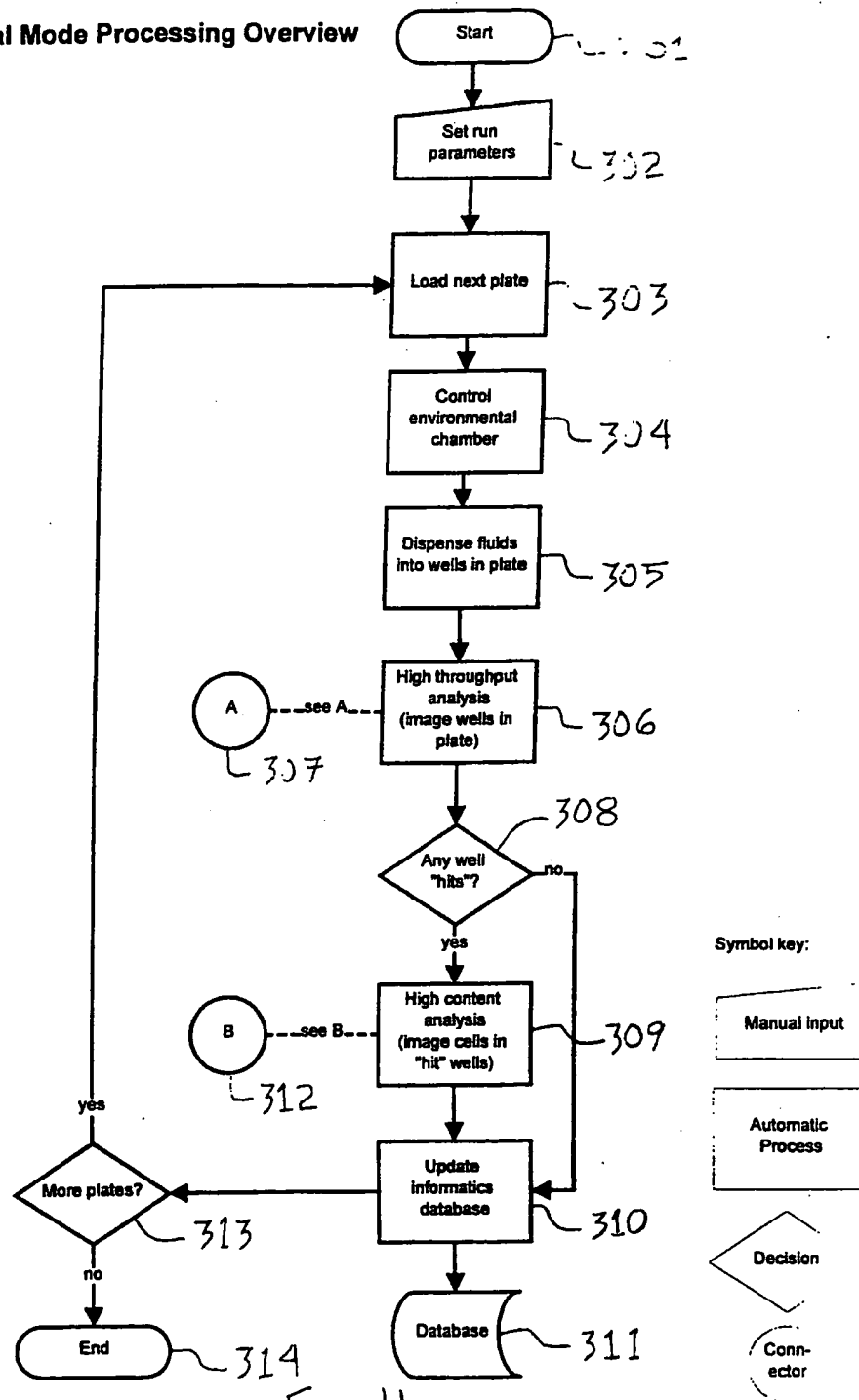


Fig. 11

## High Throughput Mode

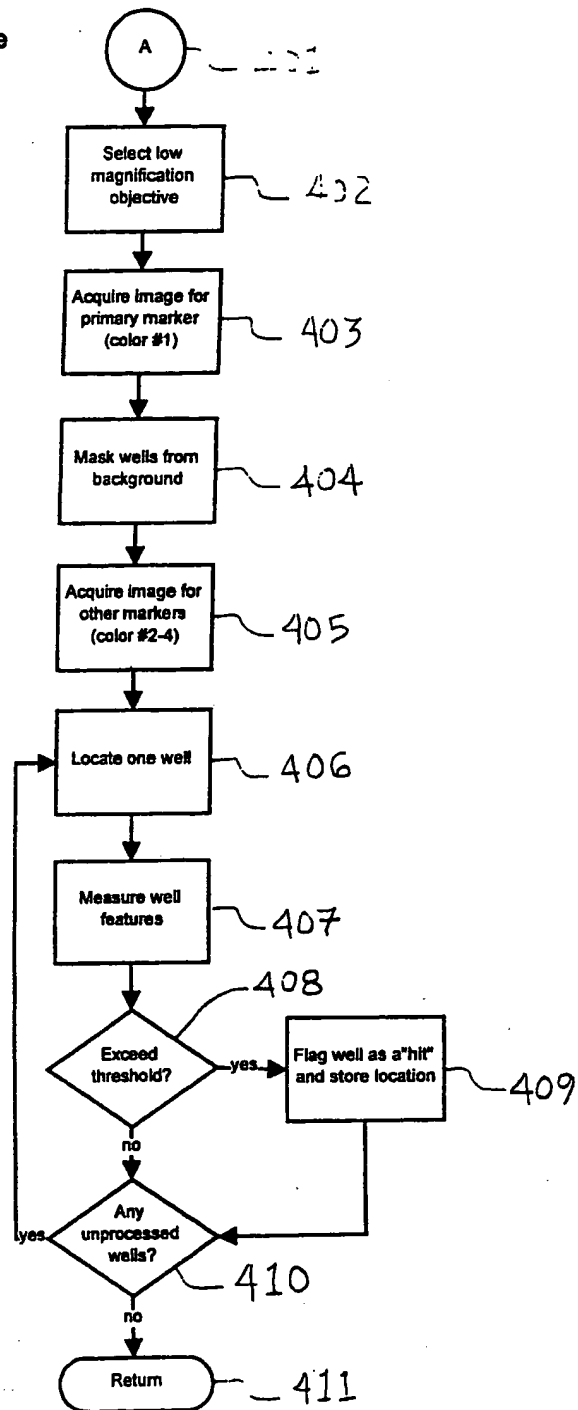


Fig. 1d

## High Content Mode

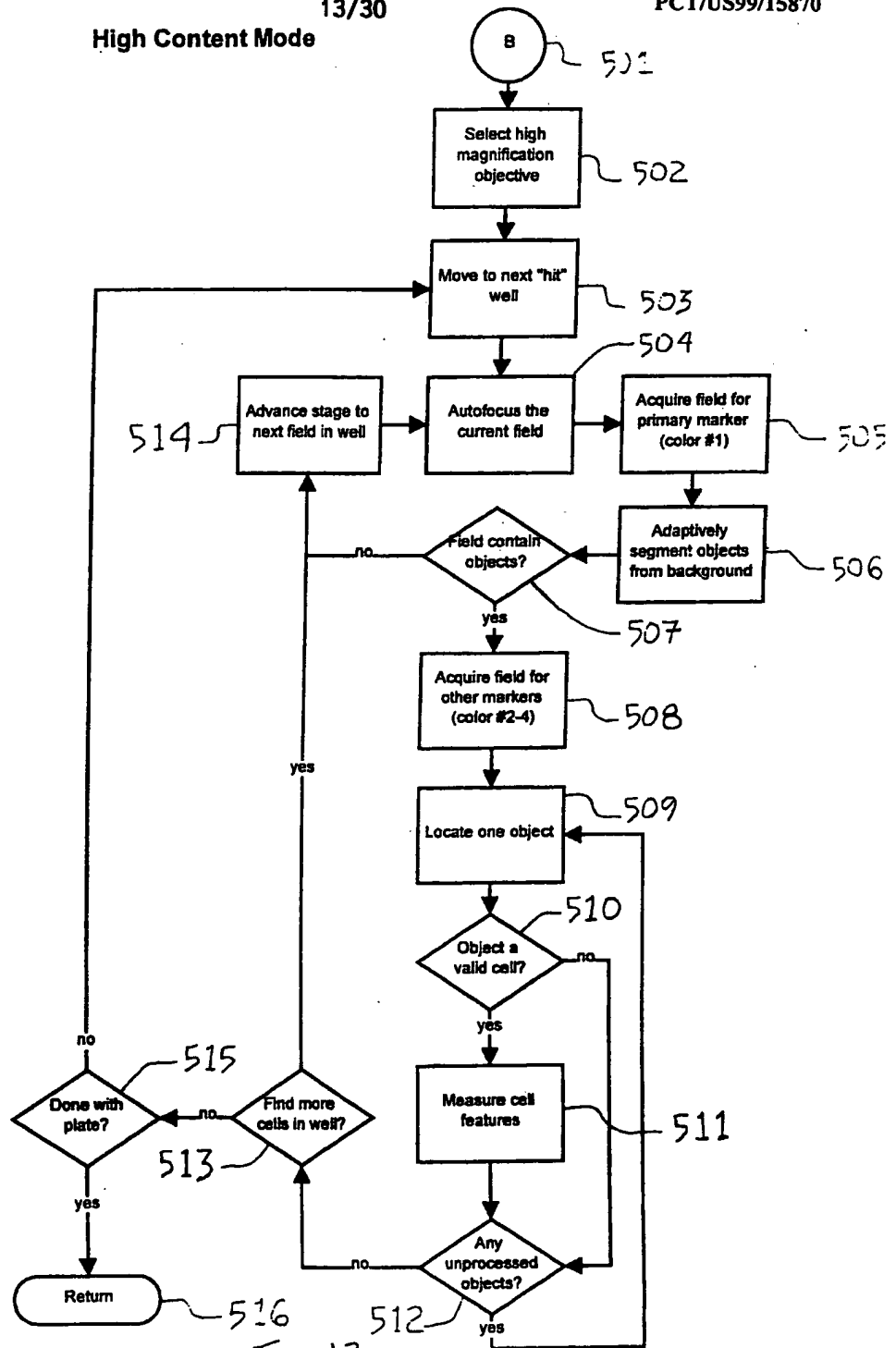


Fig. 13

## Kinetic Analysis Mode

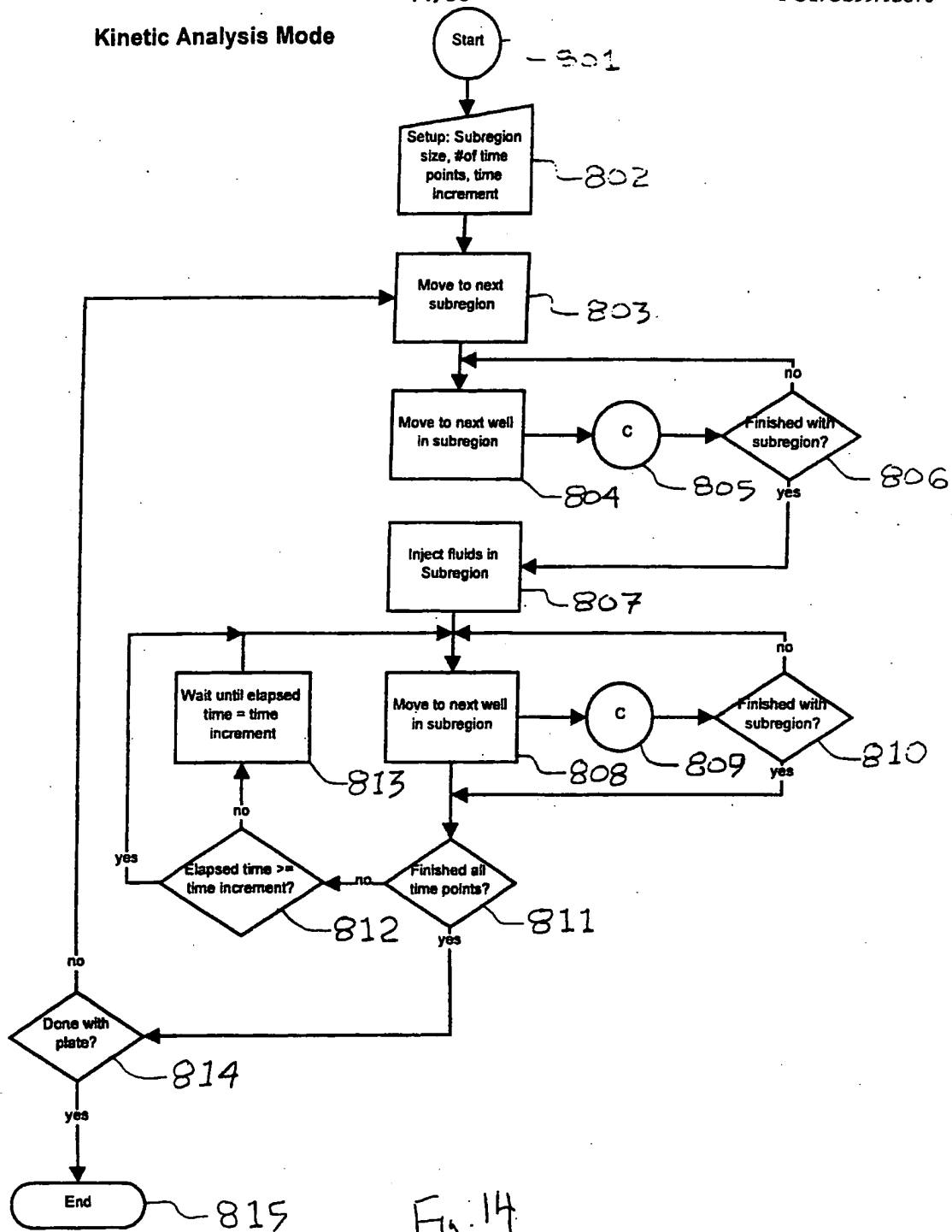


Fig. 14

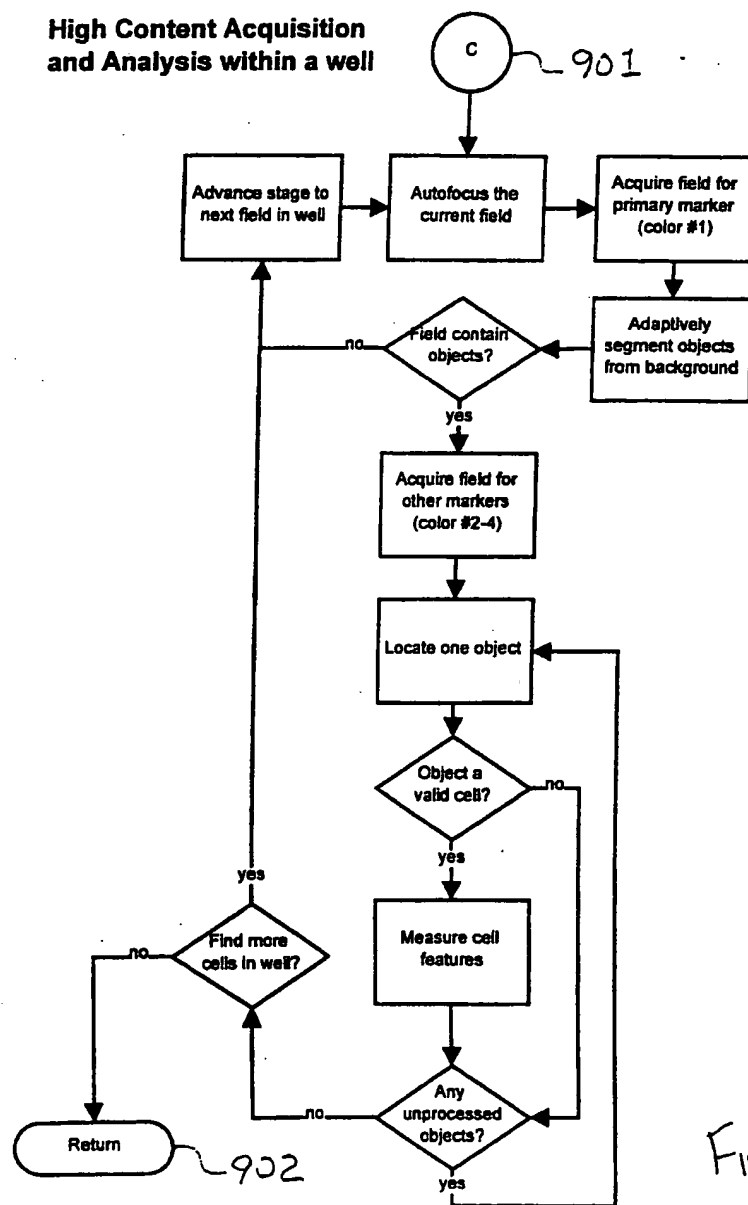
**High Content Acquisition  
and Analysis within a well**

Fig. 15

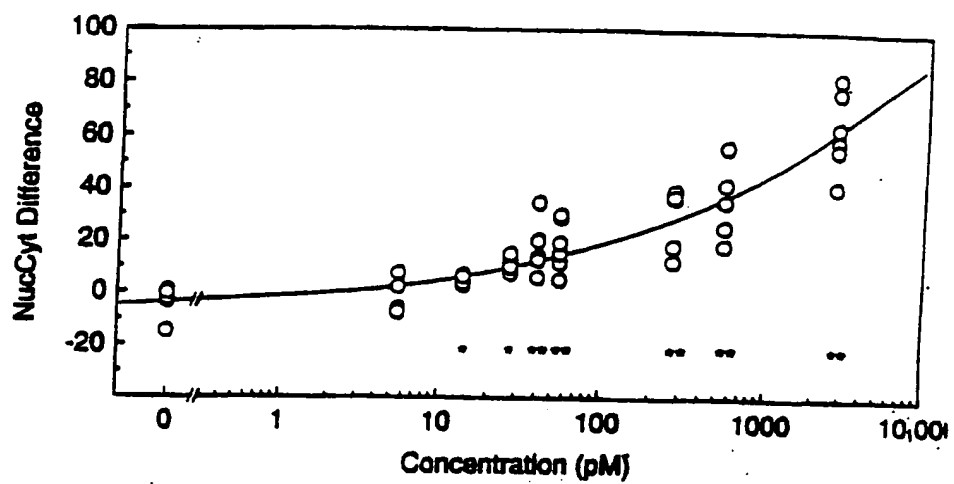
16/30  
**FIGURE 9**

Fig. 16

17/30

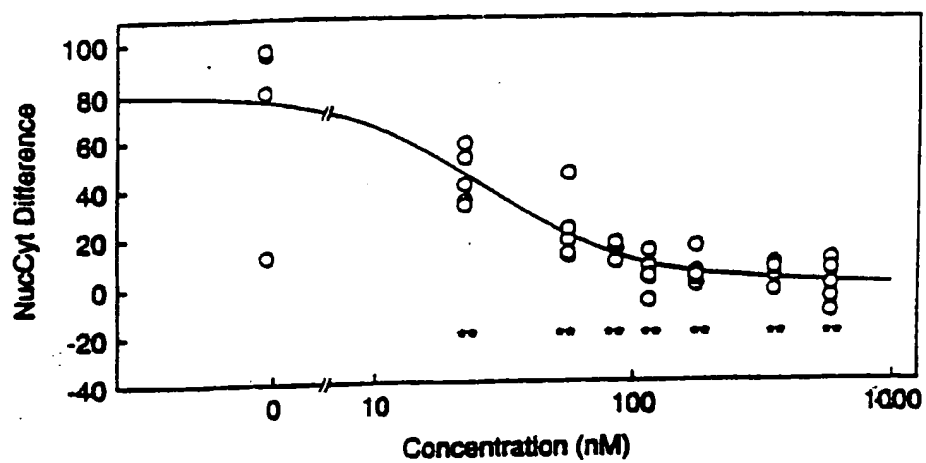
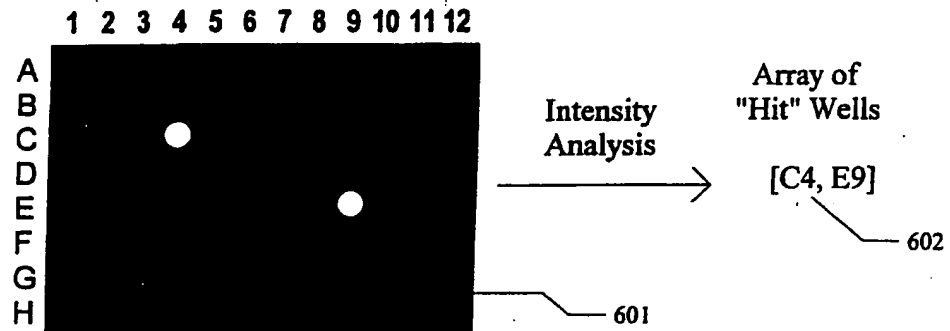
**FIGURE 10**

Fig 17





**Low Resolution  
Well Data**



**High Resolution  
Cell Data**

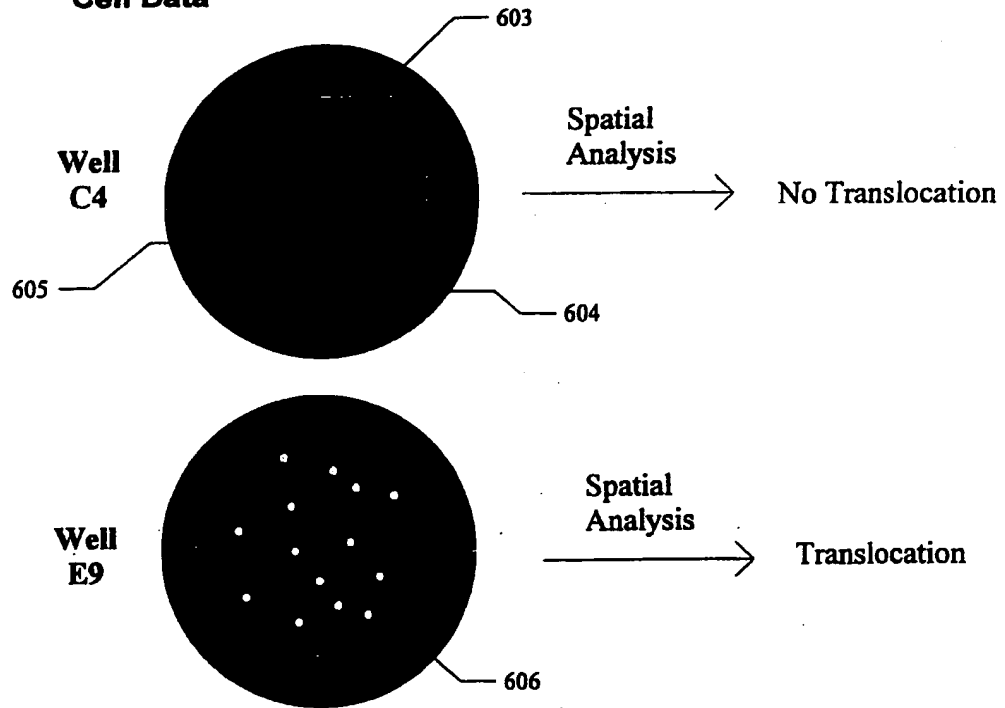


Fig 19

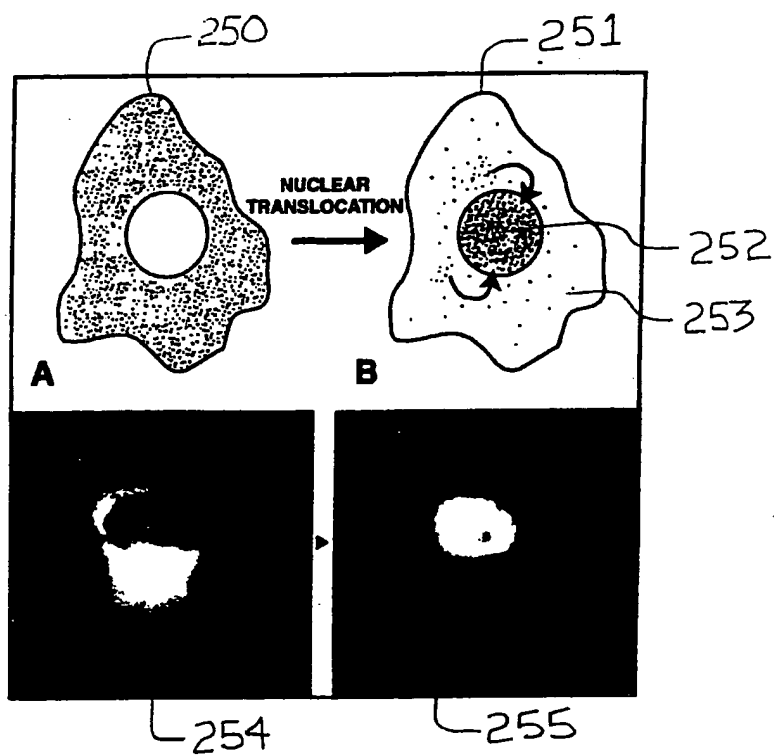


Fig. 20

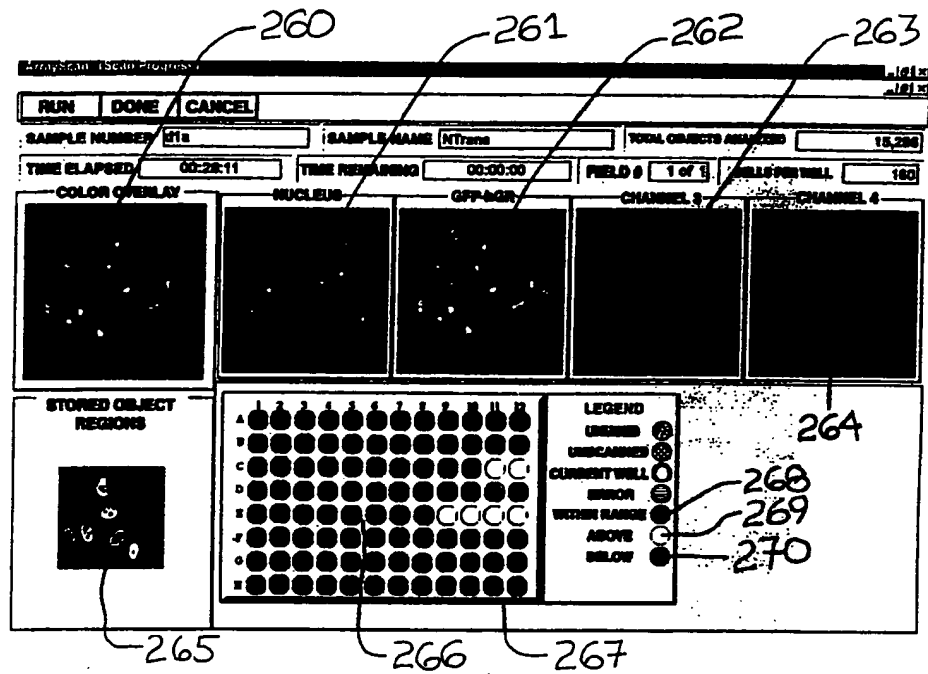
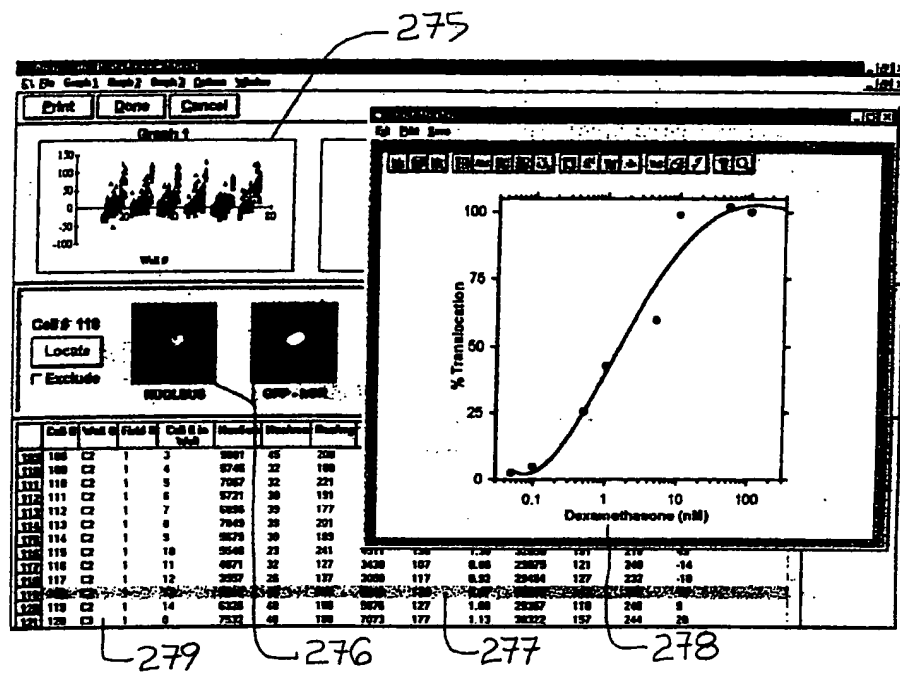


Fig 21



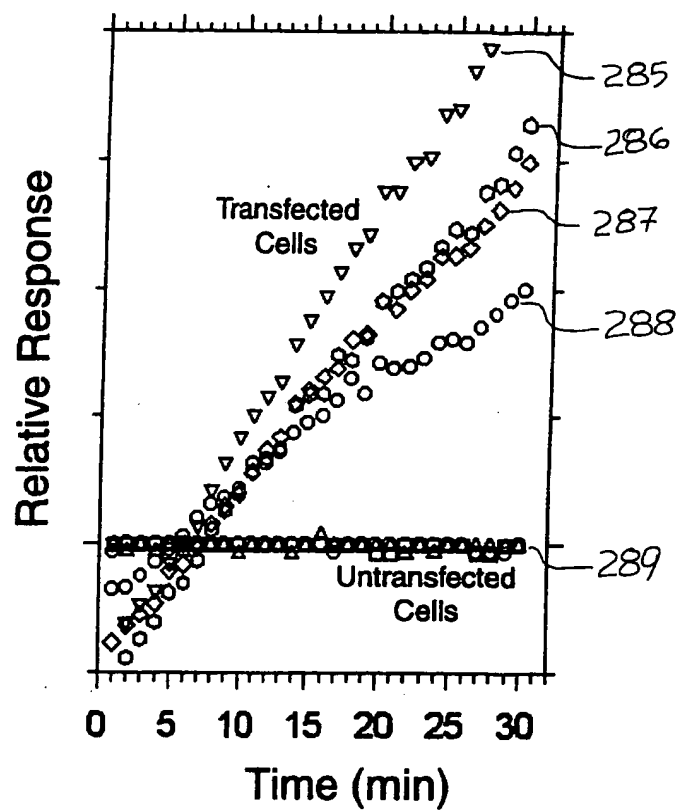


Fig. 23

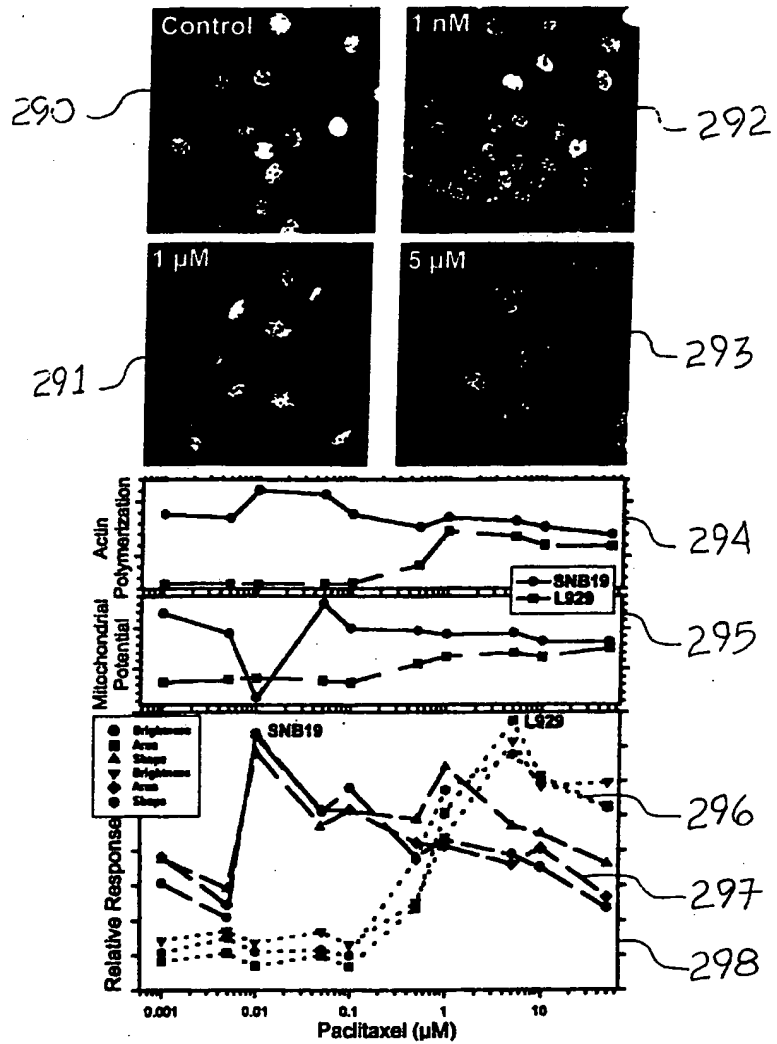
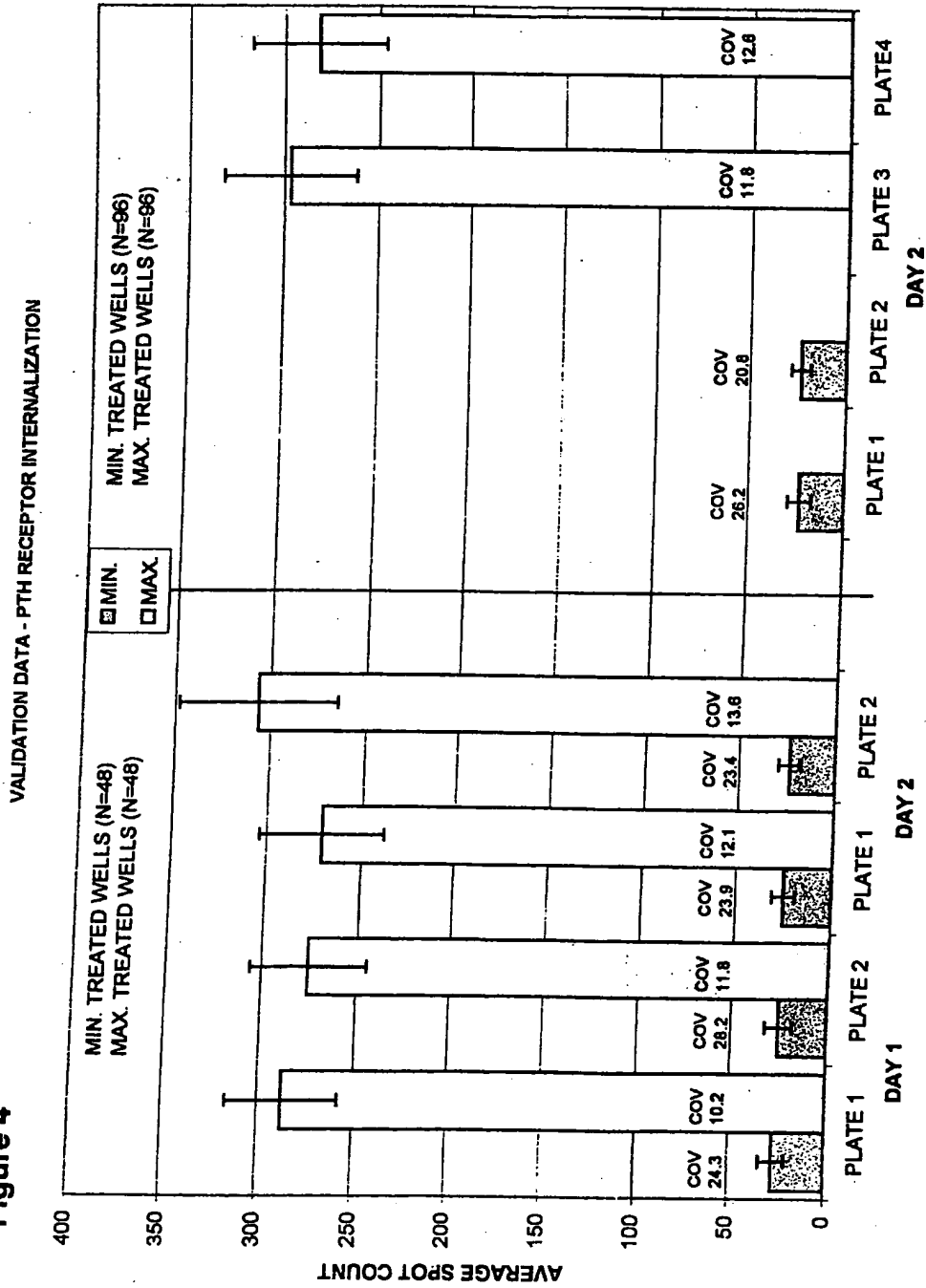


Fig. 24

5. 1

Figure 4





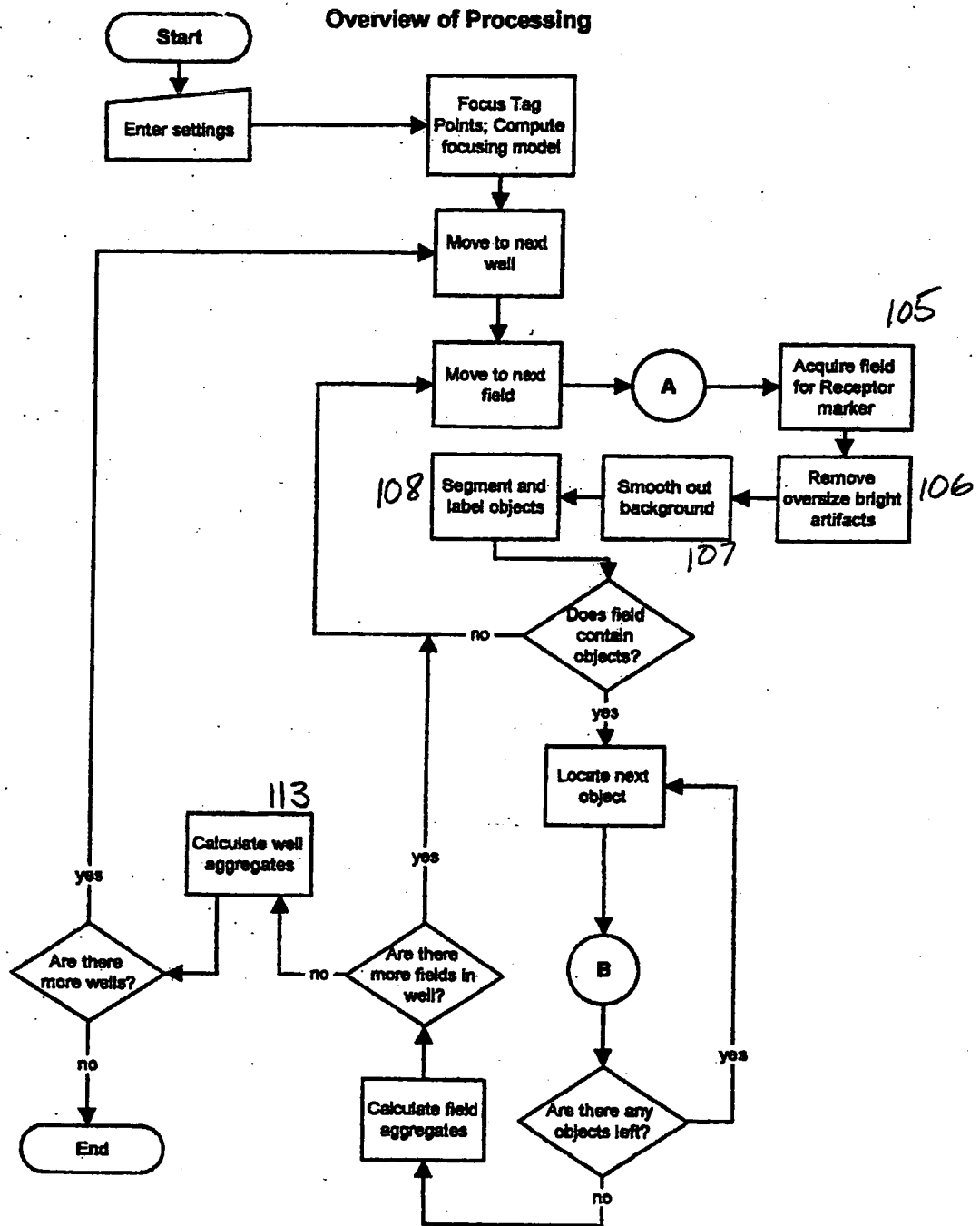


Figure 26

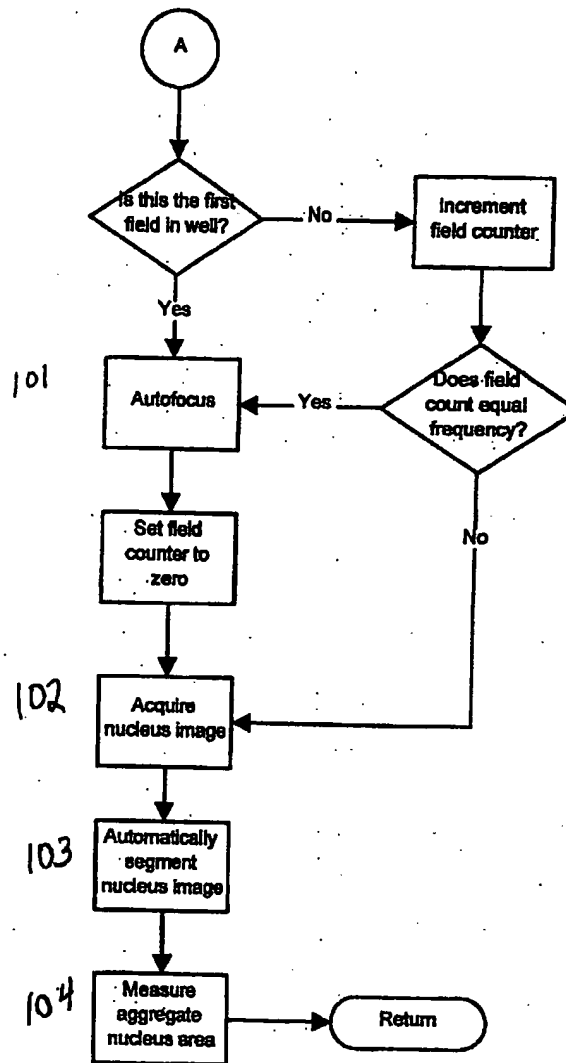
**Autofocusing Procedure**

Figure 27

## Object Processing

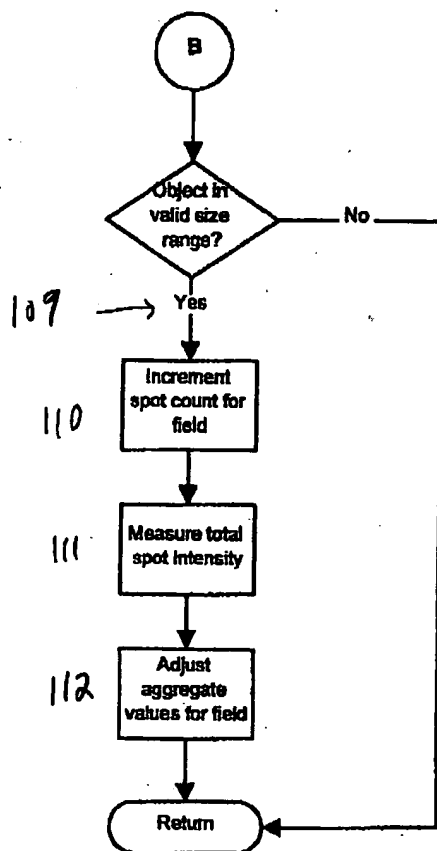


Fig 28



